Sporulation of *Stagonospora nodorum*

Rohan George Thomas Lowe, MSc.

This thesis is presented for the degree of

Doctor of Philosophy of Murdoch University.

2006

Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

.....

Rohan George Thomas Lowe

November 2006

Abstract

Stagonospora nodorum is a necrotrophic fungal pathogen that is the causal agent of leaf and glume blotch on wheat. Very little is currently known about the molecular mechanisms required for pathogenicity of *S. nodorum*, despite its major impact on Australian agriculture. *S. nodorum* is a polycyclic pathogen. Rain-splashed pycnidiospores attach to and colonise wheat tissue and subsequently sporulate within 2-3 weeks. Several cycles of infection are needed to build up inoculum for the damaging infection of flag leaves and heads, sporulation is therefore a critical component of the infection cycle of *S. nodorum*; our aim is to determine the genetic and biochemical requirements for sporulation for development of control of the pathogen. Disease progression of *S. nodorum* on wheat cv. Amery was monitored by light microscopy to determine the time point when pycnidia development began. Early pycnidia development was evident 12 days post-infection. This information was used to guide a genomics and a metabolomics based approach to determine the requirements for sporulation in *S. nodorum*. The genomics approach utilised two cDNA libraries created from sporulating and non-sporulating cultures. EST frequency was used to determine highly expressed genes under the two developmental states. Gene expression from the most highly represented genes during sporulation were confirmed using quantitative PCR. A gene encoding an arabitol 4-dehydrogenase (*Abd1*), was mutagenised, in its absence sporulation was reduced by approximately 20%. The metabolomics approach isolated metabolites from both *in planta* infection and *in vitro* growth. Rapid changes in the abundance of metabolites were detected during the onset of sporulation. Key fungal metabolites identified include mannitol and trehalose. The concentration of both mannitol and trehalose increased dramatically in concert with pycnidia formation. Both

mannitol and trehalose have also been linked to pathogenicity in filamentous fungi. Creation of deletion mutants of the gene encoding trehalose 6-phosphate synthase showed the synthesis of trehalose is required for full sporulation of *S. nodorum in planta* and *in vitro*.

Acknowledgements

Firstly, I would like to express my gratitude to my supervisors, Richard Oliver and Peter Solomon. I took a risk moving countries to do a PhD with the ACNFP, a lab I only knew by reputation. My choice was proven to be an excellent one. I have thoroughly enjoyed my time in the lab and I think I have gained experience that I would not have found in many other labs. I think the scientific and social atmosphere of a lab is determined by leadership, and I am truly glad to have studied under your guidance. I must also thank all the members of the ACNFP. I have benefited from many peoples expertise, some academic, some social, but all have helped me get to this point without a complete meltdown. I also thank Judith Lichtenzveig for expert comments on statistical methods and principles, I have managed to greatly increase my appreciation of this field, and would not have done so without your help. Outside of the ACNFP I must give special thanks to Robert Trengove and the rest of the Murdoch Separation Science Laboratory. Without your help and relentless enthusiasm I would not have learnt so much about GC-MS.

I would like to thank my friends and family for always being willing to chat about work or anything else if the lab proved too traumatic. Above all others I would like to thank Christin for always being there for me. Your love keeps me going each day, without it I would have found life infinitely more difficult.

Table of contents

List of figures

List of tables

List of abbreviations

1-P	1-phosphate
3-P	3-phosphate
6-P	6-phosphate
A	absorbance
Abd1	arabitol 4-dehydrogenase 1
acetyl-CoA	acetyl-coenzyme A
ALA	delta-aminolaevulinic acid
amp	ampicillin
AUD	Australian dollars
ave	average
bp	nucleotide base pair(s)
cDNA	complementary deoxyribonucleic acid
CE-MS	capillary electrophoresis-mass spectrometry
cv	cultivar
CZV8CS	Czapek Dox V8 juice complete supplement
d	day
DEPC	diethylpyrocarbonate
DIG	digoxygenin
DLA	detached leaf assay
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dpi	days post inoculation
E	exponential
EST	expressed sequence tag
FT-ICR	Fourier transform-ion cyclotron resonance
GABA	gamma-aminobutyric acid
GC-MS	gas chromatography-mass spectrometry
gDNA	genomic deoxyribonucleic acid
GO	gene ontology
HCA	hierarchical cluster analysis
hyg	hygromycin
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilobase pairs
LB	Luria Bertani
LC-MS	liquid chromatography-mass spectrometry
LPA	latent period assay
malonyl-ACP	malonyl-acyl carrier protein
Mb	megabase pairs
MCS	multiple cloning site
MEOX	methoxylamine
MM	minimal medium
mRNA	messenger ribonucleic acid
MST	mass-spectral tag

n	number
NMR	nuclear magnetic resonance
P Value	probability value
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
pers. comm.	personal communication
phleo	phleomycin
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
RT	retention time
SAGE	serial analysis of gene expression
SDS	sodium dodecyl sulphate
stdev	standard deviation
T6P	trehalose 6-phosphate
TCA cycle	tricarboxylic acid cycle
TMS	trimethylsilyl
TOF	time of flight
Tps1	trehalose 6-phosphate synthase 1
v/v	volume per volume
w/v	weight per volume
WPA	whole plant spray
X-GAL	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

Chapter 1 Introduction

1.1 Wheat production and fungal disease.

Wheat is the third largest cereal crop and is grown worldwide (Figure 1.1) (Lavoy, 2004). Each year approximately 600 million tonnes of wheat are produced for local and export markets. The largest producers of wheat are China, India, USA, Russia, and France (Table 1.1). Australia is a significant producer of wheat, with 22.6 million tonnes produced in 2004 (Table 1.2), worth AUD 5.6 billion (The Australian Bureau of Statistics, 2006). The state of Western Australia is the largest Australian producer (Figure 1.2). Production of this highly important crop is limited by an array of diseases caused by fungi, bacteria, viruses and nematodes. The most damaging fungal disease of wheat is rust (*Puccinia spp.)*. Other common fungal diseases are: leaf and glume blotch (*Stagonospora nodorum*), scab and ear blight (*Fusarium* spp.), take-all (*Gaeumannomyces graminis*), septoria blotch (*Mycospharella graminicola*), powdery mildew (*Erysiphe graminis*), and tan spot (*Pyrenophora tritici-repentis)*. The study of fungal plant pathogens is an economically important area; this study focuses, *Stagonospora nodorum*, cause of leaf and glume blotch on wheat.

1.2 Stagonospora nodorum.

Phaeosphaeria nodorum ((E. Müller) Hedjaroude., Sydowia 22(1-4): 79 (1968). (Hedjaroude, 1969) or *Stagonospora nodorum* (Berk.) Castellani & E. G. Germano, Annali Fac. Sci. agr. Univ. Torino 10: 71 (1977) is a necrotrophic fungal pathogen of wheat. An abbreviated taxonomy is as follows: phylum, ascomycota; class, ascomycete; class, dothideomycetes; order, pleosporales. Synonyms include *Leptosphaeria nodorum,* and *Septoria nodorum*. The teleomorph is named *Phaeosphaeria nodorum*. The anamorph, *Stagonospora nodorum*, has a haploid

Figure 1.1

Table 1.1

Table 1.2

Figure 1.2

genome with 14-19 chromosomes (Cooley and Caten, 1991) and a genome size of 37 Mb (The Broad Institute, 2005) .

1.3 Necrotrophic fungal pathogens.

Necrotrophic pathogens are conventionally defined as those that kill the host tissue before aggressively invading and feeding from it. This is in contrast to biotrophic pathogens which tend to be obligate, and feed from living plant cells. Hemi-biotrophs are pathogens with distinct, temporally separate biotrophic and necrotrophic stages (Oliver and Ipcho, 2004). The divisions drawn between pathogens are not perfect, and frequently a pathogen is reported as belonging to more than one class. Currently, the criteria for classifying fungal pathogens as either biotrophic, hemi-biotrophic or necrotrophic are being clarified. New criteria include the presence of haustoria, to biotrophy, and the plant defence response differentiating classes of pathogen (Oliver and Ipcho, 2004). A key characteristic of fungal nectrotrophs is the export of toxins, either host-specific or non-specific, to kill the host tissue ahead of colonisation (Liu *et al.*, 2004).

1.4 Stagonospora nodorum and wheat life cycle

S. nodorum is usually isolated from wheat. Isolates have been recovered from barley, but these isolates are usually unable to infect wheat and *vice versa*. Passage through alternate graminaceous hosts is known to decrease its pathogenicity on wheat (Hann and Griffiths, 1976).

The inoculae for epidemics is thought to be from infected seed or wind-borne sexual ascospores (Arseniuk *et al.*, 1998; Shah *et al.*, 1995; Shah and Bergstrom, 2000).

Dispersal over large distances primarily occurs via wind-borne ascospores during winter. Ascospores develop from growth on stubble and refuse from the previous year's crop and conditions of rainfall (>1 mm), high humidity (75-95 %) and low temperature (0 ºC-20 ºC) initiate their release from perithecia (Arseniuk *et al.*, 1998; Bathgate and Loughman, 2001). Shah *et. al*. (2001) analysed the genotypes present in a field epidemic and found that genetically diverse populations were present before the wheat canopy formed, indicating many different ascospore-initiated inocula were present. They also noted that once disease had been initiated in a new area, it propagated locally via clonal asexual reproduction. It has been estimated that 2-4 cycles of asexual reproduction would be required before infection could spread to the glume (Shah and Bergstrom, 2002). Although the sexual phase of *S. nodorum* has been recognised as a critical part of disease outbreak, it is poorly understood.

The process of infection follows the usual stages for necrotrophs: Germination and penetration followed by collapse of the epidermal and mesophyll cells of the host. The hyphae spread rapidly throughout the tissue, but avoid the vascular bundle (Solomon *et al.*, 2006c; Solomon *et al.*, 2006f). Full colonisation is marked by the development of asexual sporulation structures, termed pycnidia. Pycnidia produce masses of asexual pycnidiospores, which are released onto the leaf surface when mature. Pycnidiospores are can be spread a short distance around the plant by rain splash. Multiple cycles of infection and asexual reproduction are required to infect the upper plant canopy, where the most damaging infection occurs on the flag leaf and glume (Figure 1.3).

Spores germinate on the leaf surface, usually within 3-12 hours of deposition (Solomon *et al.*, 2006f). Germination is usually unipolar, and occasionally bipolar. Hyphae

quickly spread and traverse the surface of the leaf, sometimes over relatively large distances before a penetration attempt is made. Penetration can occur via stomatal openings or directly through the cuticle, but preferential growth toward stomata has been observed. Host cell chlorosis and death occurs around penetration sites, often in a large area around the hyphae, indicating only minor interaction is required to initiate cell death of the host. Penetration via the cuticle sometimes involves hyphal swellings that most closely resemble hyphopodia, but these structures are not required for penetration (Solomon *et al.*, 2006f). As chlorosis spreads from the site of in infection, a macroscopically visible oval tan lesion is formed.

Asexual sporulation is the most common and well understood form of reproduction in *S. nodorum*. Pycnidia can form *in planta* by seven days post-infection under ideal conditions (Figure 1.4). Infections proceed most quickly at high humidity and at 20 ºC. Pycnidia form within the lesion, but usually slightly away from the initial site of penetration. Pycnidia begin development as hyphal aggregations, without colouration, but quickly swell and differentiate to become melanised, brown, ovoid structures just beneath the leaf surface (Douaiher *et al.*, 2004). Before pycnidiospores are released, an ostiole forms on the exposed surface of the pycnidium. The ostiole is a conical projection,

Figure 1.3

Figure 1.4

from which the pycnidiospores are released. The exudate is a mixture of pycnidiospores and cirrus, appearing as a thick glistening suspension on the surface of the leaf. Cirrus has been defined as "a gel composed of proteinaceous and saccharide compounds" (Cunfer, 1999). The initial infection is characterised by distinct small lesions spread randomly on the leaf surface. As infection progresses these lesions merge and eventually the remainder of the leaf will quickly senesce. Once this occurs, hyphae spread rapidly throughout the remaining tissue and asexual sporulation occurs *en masse*. Pycnidiospores will travel short distances about the plant architecture by water splash, and dilution of the cirrus is required before germination will occur (Cunfer, 1999). Several rounds of the asexual growth cycle are required before an infection will reduce the yield of grain from the plant. This occurs either via reduction of photosynthetic activity in the plant, particularly notable in the flag leaf, or via colonisation and damage to the glume directly (glume blotch) (Figure 1.5).

1.5 Worldwide distribution

S. nodorum is an important necrotroph because of its potential to cause severe crop damage, and its prevalence in several important wheat growing regions, including central and northern states of the USA and in Western Australia (WA).

Its impact on grain yields are high, and may cause losses of up to 18-31 % (Bhathal *et al.*, 2003). *S. nodorum* is of particular importance in WA because of its dominance of Australian wheat production (Table 1.2) is coupled with an unusually high incidence of the disease. A survey of Australian plant pathologists estimated that *S. nodorum* caused a 5-15% reduction in wheat yield in Western Australia (Fig 1.2 B) (Brennan and Murray, 1998).

Figure 1.5

S. nodorum leaf and glume blotch used to be the dominant wheat disease throughout Europe during the 1970s and 80s, but has recently been supplanted by *Septoria tritici*. Climate change is now a phenomenon almost universally acknowledged among scientists (Oreskes, 2004), this suggests that climatic conditions that either support or deny access to fungal pathogens are likely to change in all wheat growing regions. These unpredictable long-term changes in local climate could dramatically alter the regions where *S. nodorum, or indeed any fungal pathogen,* is a problem, in a similar manner to the sudden reduction in *S. nodorum* epidemics in Europe (Bearchell *et al.*, 2005). Therefore, it is vitally important to fully understand the biology of these diseases so that effective control strategies are developed for currently affected wheat growing regions and those that may soon be affected (Garrett *et al.*, 2006).

1.6 Control of leaf and glume blotch.

The main methods of control are through farm management practises, use of partially resistant wheat cultivars, and application of fungicides to affected crops. Recently adopted agronomic techniques, such as minimum tillage agriculture, have helped to reduce soil erosion and improved water use efficiency, but by increasing the amount of stubble left on fields have increased pathogen loads on subsequent crops.

Certain cultivars of wheat provide genetic resistance to leaf and glume blotch. Only partial resistance has been described, and its inheritance has been shown to be a quantitative trait (Czembor *et al.*, 2003). The cloning of resistance genes in wheat is very difficult due to large genome sizes; furthermore, resistance to leaf blotch and glume blotch may involve different genes (Solomon *et al.*, 2006c).

Correct application of fungicide usually controls outbreaks of leaf and glume blotch, however, fungicides are expensive and may not be economically beneficial (Stover *et al.*, 1996). The main types of fungicides currently in use are ergosterol biosynthesis inhibitors, including difenoconazole, triadimenol and mitochondrial respiration inhibitors, the strobilurins (Dutzmann *et al.*, 2004). Strobilurins have shown additional benefit by increasing the lifespan of the flag leaf (Gooding *et al.*, 2000). However, resistance to strobilurins has been reported (Morzfeld *et al.*, 2004). Sequencing of the mitochondrial cytochrome b gene in resistant isolates suggested that the a single point mutation (resulting in Gly to Ala substitution) was associated with resistance to the two strobilurin fungicides (Ishii *et al.*, 2001). Morzfeld *et. al.* (2004) reported that increased resistance to azoxystrobin was obtained by *Septoria nodorum* after 4-5 generations of growth *ad planta*, but not *in vitro*.

1.7 Molecular genetics in *Stagonospora nodorum*.

Molecular genetics have proved to be the dominant path for elucidation of pathogenicity mechanisms as sexual crosses are difficult to induce in a lab environment. Reports of ascospore production have been published. In one example the best conditions were: Incubation of cultures for 60 days at 10ºC, with a 12 hour photoperiod under near-UV light (Halama and Lacoste, 1991). Because of difficulty inducing the sexual cycle, crosses are not a common part of lab procedures. Conversely, the asexual cycle is readily completed in the lab both *in planta* and *in vitro*. *S. nodorum* grows easily on many defined and complex media. Asexual pycnidiospores can easily be produced *in vitro* within 7 days, and the yield of spores is enhanced by exposure of cultures to near-UV light.

The haploid nature of the *S. nodorum* genome makes it a particularly attractive system to perform genetic manipulations at the molecular level. *S. nodorum* was first transformed by Cooley *et. al.* (1988). Targeted homologous recombination was achieved in 1998 by Howard *et. al.* (1999), and requires only 1000 to 1500 bp of homologous DNA (Solomon *et al.*, 2003). Recent work suggests that the rate of recombination increases when constructs with entirely homologous ends are used (Peter Solomon, pers. comm.). Several selectable markers have been described; for example, nitrate resistance, hygromycin resistance, and phleomycin resistance. Heterologous expression of genes is possible with the commonly used *A. nidulans GpdA* promoter and *TrpC* terminator. Three expressed-sequence tag (EST) libraries have been made from cultures grown on wheat cell-wall media, oleate media and from a late infection/sporulation stage in wheat (this study). A recent development of great significance was the release of the *S. nodorum* SN15 genome sequence. Whole-genome shotgun sequencing was performed by the Broad Institute using two plasmid libraries (4 kb and 10 kb clones) and a fosmid library. The sequence is publicly available online at www.broad.mit.edu and GenBank. Sequence coverage has been estimated at > 10x, and the initial assembly is of a very high standard. Genome statistics are as follows, total genome size, 37.1 Mb, 496 contigs among 109 supercontigs, with an average contig length of 74.7 kb. A basic annotation of the assembly has been performed, using FGENESH, FGENESH+, GENEID, and GENEWISE gene finding software, blastX comparison to the non-redundant protein database and 320 manually annotated genes. The total number of genes annotated was 16597. The average gene length was 1309 bp, and the average gene density was one gene every 2243.5 bp. The average number of exons per gene was 2.65. The current annotation is thought to overestimate the true number of genes, and an updated annotation is currently being prepared (Richard

Oliver, pers. comm.). The body of work related to this organism is likely to grow significantly in size over the next few years as researchers take advantage of this valuable resource.

1.8 Molecular biology of pathogenicity of *Stagonospora nodorum*.

S. nodorum is not only a major wheat pathogen, but also a model necrotroph. It is easily cultured in the lab, it has a rapid disease cycle (one week in ideal conditions) and targeted genetic manipulations are performed relatively easily for a filamentous fungus. Despite these encouraging attributes, studies of *S. nodorum* using molecular genetics are few in number. The first published account of molecular investigation of the pathogenicity of *S. nodorum* was by Howard *et. al.* in 1999. The gene encoding nitrate reductase (*Nia1*) was replaced with the hygromycin B resistance gene, *Hph*, under the control of the GpdA promoter and TrpC terminator. The experiment demonstrated that homologous recombination was a useful method for testing possible fungicide targets in *S. nodorum*. While transformants were recovered, pathogenicity on wheat was not affected. Cooley *et. al.* published an account of the targeted deletion of the gene encoding 3-isopropylmalate dehydrogenase (*LeuA*) (Cooley *et al.*, 1999). The mutants were auxotrophic for leucine, and were non-pathogenic on wheat. Induced auxotrophy via gene knockouts is a useful tool to test possible metabolic weaknesses in fungi. The gene encoding ornithine decarboxylase (*Odc1*) has been shown to be essential for pathogenicity (Bailey *et al.*, 2000). Interestingly, the same study found that *SnOdc1* was able to restore sporulation in an *Aspergillus nidulans* strain lacking *AnOdc1*, suggesting a link between polyamine production and sporulation as well as pathogenicity. Several studies have been published where the deletion of a particular

gene, thought to be involved in pathogenicity, failed to change the behaviour of the pathogen. *Snp1* - trypsin-like protease (Bindschedler *et al.*, 2003), *Ptr2* - di/tripeptide transporter (Solomon *et al.*, 2003) and *Gox1* - glyoxalase I (Solomon and Oliver, 2004) are examples where pathogenicity was not affected by this loss, despite clear demonstration of the loss of a particular function in the mutants. Casual inquiries of researchers in the field indicates that the frequency of "pathogenicity normal" mutants is far greater than the literature would suggest.

Returning to the topic of induced auxotrophy, there are another two published examples where loss of function auxotrophs were severely affected in pathogenicity or sporulation. Deletion of the gene *Als1*, encoding Delta-aminolaevulinic acid (ALA) synthase, resulted in strains auxotrophic for ALA and unable to cause disease on wheat leaves (Solomon *et al.*, 2006a). Removal of mannitol 1-phosphate dehydrogenase activity by insertional mutagenesis of *mpd1* also resulted in mutants that could still form lesions on wheat leaves, but were unable to produce any asexual spores *in planta*. The reduction in sporulation *in planta* was linked to reduced mannitol levels in *mpd1* strains when grown on glucose as the sole carbon source.

Mutants deficient in catabolic pathways have also been described. The use of stored lipid as an energy source for asexual spores has been investigated. The gene encoding malate synthase (*Mls1*) has been deleted by targeted mutagenesis (Solomon *et al.*, 2004a). The *mls1* mutants were unable to cause disease on wheat leaves, due to the inability of spores to germinate. The germination failure was explained by the inability to break down the stored lipid, as supplementation of glucose and sucrose fully restored germination and pathogenicity. The study showed that gluconeogenesis and the

glyoxylate cycle were required for pathogenicity. Also, more recent studies on the mannitol cycle in *S. nodorum* have shown that a double mutant lacking both *Mpd1* and *Mdh1* resulted in almost total loss of endogenous mannitol (Solomon *et al.*, 2006e). Under these conditions *mpd1mdh1* mutants could no longer use mannitol as sole carbon source, and were unable to sporulate *in vitro* or *in planta*. Sporulation could be restored by the addition of exogenous mannitol.

The disruption of signal transduction pathways has been studied in *S. nodorum.* Heterotrimeric g-protein signaling was studied, by the disruption of the gene encoding the g-alpha subunit (*Gna1*) (Solomon *et al.*, 2004b). These mutants were severely compromised and failed to cause disease on wheat leaves or sporulate. In addition, another study targeted the mitogen-activated protein kinase (MAPK) signaling cascade, by deleting the gene encoding MAP kinase, *Mak2* (Solomon *et al.*, 2005b). Mutants lacking *Mak2* were unable to sporulate *in vitro* or *in planta* and had a near-total loss of pathogenicity. These two examples show the critical importance of signaling pathways in the activation of reproduction and pathogenicity systems.

Host specific toxins have been investigated in *S. nodorum*. Host-specific toxins confer virulence to pathogens on hosts that contain the corresponding susceptibility locus. In this case, *ToxA* confers virulence on *Tsn1*-containing wheat. Deletion of ToxA in SN15 and Sn2000 halted expression of ToxA and eliminated the correlation of virulence with the presence of the Tsn1 locus in wheat. Friesen *et. al.* (2006) reported the inter-specific gene transfer of a host-specific toxin from *S. nodorum* to *Pyrenophora tritici-repentis*. The virulence gene-transfer event between *S. nodorum* and *P. tritici-repentis* is the first evidence-supported case to be described in the fungal kingdom.

1.9 Sporulation in the filamentous fungi.

Sporulation is an essential part of the lifecycle of fungi. Without sporulation, growth would be limited to the immediate area, with no chance of dissemination to new environments or survival in challenging environments. The switch from growth as hyphae to the development of sporulation structures can be triggered by various environmental stimuli. Examples of reported stimuli include nutrient supply, light exposure, pH, and metal ions (Calvo *et al.*, 2002). Sexual development of *Fusarium graminearum* is stimulated by synthesis or application of the mycotoxin zearalenone (Wolf and Mirocha, 1977), while the cyclin-like gene *Fcc1* has an influence on production of the mycotoxin fumonisin and asexual sporulation. In *Aspergillus nidulans*, sterigmatocystin synthesis is correlated with conidiation (Wilkinson *et al.*, 2004).

The study of sporulation in filamentous fungi is dominated by research of *Neurospora crassa* and *Aspergillus nidulans*. Filamentous fungi such as *Aspergillus nidulans*, *Neurospora crassa* and *S. nodorum* can be prevented from sporulating by growth in submerged culture. In *A. nidulans*, hyphae must be exposed to an air-water interface before either asexual or sexual sporulation will occur (Adams and Wieser, 1999). By exposing hyphae grown in submerged culture to air, it has been shown that there is a minimum period of growth of at least 18 hours required before hyphae become competent for sporulation (Yager *et al.*, 1982). Once competency has been achieved, it takes another five hours growth exposed to air before spore formation begins. If hyphae are exposed to air before competency is achieved, the time to sporulation increases accordingly. These results suggest that induction of sporulation in *A. nidulans* is only triggered by aspects of the life-cycle and not by a nutrient-limitation or unfavourable

environment. In fact, if the growth medium beneath a surface growing colony is continually replaced, sporulation is not repressed, showing nutrient starvation is not essential for sporulation (Pastushok and Axelrod, 1976). However, it appears that while nutrient stress is not essential, both carbon and nitrogen stress can act as the trigger in the absence of other signals, once competency have been acheived (Lee and Adams, 1996).

Wild-type *A. nidulans* strains contain the velvet gene (veA^{+}), which prevents activation of conidiation until the colony is exposed to red light. Most lab strains have the *veA1* mutation, and will readily sporulate without stimulation by red light. This suggests the *VeA* product represses sporulation.

External stimuli are often transmitted into the cell via g-protein coupled receptors (GPCRs) on the cell exterior. These receptors transduce the signal to effector molecules within the cell via heterotrimeric g-protein complexes and downstream signal transduction cascades. These systems are the subject of intense study, but in relation to mycotoxin production and sporulation of fungi the most studied are the *Aspergillii*. The current research in this field was recently reviewed by Brodhagen and Keller (2006). There are three main pathways that regulate sporulation in *A. nidulans*; the FadA, the FluG, and the GanB pathways (Figure 1.6).

The FadA pathway begins with signalling from an unidentified GPCR on the cell surface which stimulates the FadA-GpgA-SfaD containing heterotrimeric g-protein complex. FadA dissociates from the other two subunits and activates production of cyclic-AMP (cAMP) which stimulates hyphal growth via protein kinase A (PkaA)

(Shimizu *et al.*, 2003). Activated FadA results in free GpgA-SfaD (the g-beta and g-gamma subunits), which can activate AflR, a transcriptional regulator of aflatoxin biosynthesis. Free GpgA-SfaD also repress LaeA, (a global repressor of gene cluster transcription) and BrlA, (a transcriptional activator of asexual sporulation effectors) (Seo and Yu, 2006). The effect of FadA activation is increased hyphal growth, repression of sporulation and increased mycotoxin production (Shimizu and Keller, 2001). The second major pathway is the FluG pathway. This begins with the protein FluG producing an unknown chemical signal to induce sporulation. The FluG signal is detected by an unknown receptor, which activates FlbA, a repressor of FadA (Hicks *et al.*, 1997). The effect of FlbA activation is stimulation of BrlA (and thus asexual sporulation) and repression of mycotoxin production (Shimizu *et al.*, 2003). The third pathway begins with an unidentified GPCR activating a heterotrimeric g-protein containing the g-alpha subunit GanB. The activated GanB subunit then represses BrlA and stimulates effectors for germination, carbon sensing and stress responses (Chang, 2003). Cyclic AMP signalling via heterotrimeric g-protein complexes has been shown to regulate sporulation in many fungi, including *N. crassa* (Ivey *et al.*, 1996), *A. nidulans* (Hicks *et al.*, 1997), *M. grisea* (Liu and Dean, 1997), *Cryphonectria parasitica* (Parsley *et al.*, 2003), *Ustilago maydis* (Kruger *et al.*, 1998), *Trichoderma atroviride* (Reithner *et al.*, 2005), *Cryptococcus neoformans* (Lee *et al.*, 2003), *S. nodorum* (Solomon *et al.*, 2004b), *Cochliobolus heterostrophus* (Horwitz *et al.*, 1999), *Penicillium marneffei* (Zuber *et al.*, 2003), *Botrytis cinerea* (Gronover *et al.*, 2001). The wide range of fungi that require g-protein signalling shows the central importance of this type of signalling.

Figure 1.6

While g-protein signalling has been shown to be required for sporulation, it is also a requirement for many other cellular functions. This makes it difficult to dissect the various functions. This study will focus on the effectors downstream of these signal transduction pathways. This approach is intended to allow the specific requirements of sporulation to be resolved.

1.10 Summary

S. nodorum is both a potent pathogen and one that can be easily manipulated in the lab. The disease it causes is damaging and affects the third largest cereal crop worldwide. The epidemiology shows that sporulation is a critical part of the lifecycle, required for both dissemination of genetic diversity and for localized colonization of the wheat plant. Current fungicide control strategies for *S. nodorum* focus on the prevention of spore germination and penetration, ignoring the dissemination of disease by sporulation. The loss of the asexual cycle would effectively prevent disease progression in a field situation, and dramatically reduce any yield penalty. This study concentrates on revealing aspects of the biology of sporulation by *Stagonospora nodorum*. In particular, this study will focus on genes that are downstream effectors of sporulation development. By determining genes that are required for sporulation, new control strategies can be devised to attack this aspect of pathogenicity.

Chapter 2

General materials and methods

2.1 General reagents

Chemicals and general reagents were obtained from Merck or Sigma, unless otherwise noted.

2.2 Media

All media were dissolved in sterile H_2O and autoclaved at 121°C, 15 psi for 20 minutes unless otherwise mentioned.

2.2.1 Luria-Bertani medium.

Luria-Bertani (LB) broth contained; 0.85 mM NaCl, 10 g/L Bactotryptone (Difco), 5 g/L yeast extract (Difco) (pH 7.0–7.5). LB agar contained an additional 10 g/L agar (Bio-Rad).

2.2.2 SOC medium.

SOC medium contained; 2% (w/v) Bactotryptone (Difco), 0.5% (w/v) yeast extract (Difco), 20 mM glucose, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2.5 mM KCl.

2.2.3 CZV8CS solid medium.

CZV8CS solid medium contained; 45.4 g/L Czapek-Dox agar, 200 mL/L centrifuged V8 juice, 15 g/L agar, (pH 6.0.). 5 mL/L filter-sterile complete supplement (CS) (2.3.9) was added post-autoclaving.

2.2.4 CZV8CS broth.

CZV8CS broth contained; 45.4 g/L Czapek-Dox liquid broth, 200 mL/L centrifuged V8 juice, (pH 6.0.), 5 mL/L filter-sterile complete supplement (2.3.9) was added post-autoclaving.

2.2.5 Minimal medium.

Minimal medium (MM) broth contained; 30 g/L Sucrose, 2 g/L $NaNO_3$, 1 g/L K_2HPO_4, 10 mL/L 100X Trace solution (2.3.10). Solid minimal medium contained an additional 15 g/L agar (pH 6.0).

2.2.6 Benzimdazole agar

Benzimdazole agar contained 10 g/L agar and 0.15 g/L benzimadazole.

2.2.7 CZV8 protoplast medium.

CZV8 protoplast medium contained 45.4 g/L Czapek-Dox agar, 10 g/L Agar, 182.2 g/L Sorbitol, 200 mL/L Centrifuged V8 juice. pH 6.0.

2.2.8 CZV8 protoplast top agar.

CZV8 protoplast top agar contained 45.4 g/L Czapek-Dox liquid broth, 7.5 g/L Agar, 182.2 g/L Sorbitol, 200 mL/L Centrifuged V8 juice. pH 6.0.

2.3 Media additions

2.3.1 Ampicillin

Stock ampicillin solution contained; 0.29 M ampicillin. A working concentration of 0.29 mM was used. The stock solution was stored at -20°C.

2.3.2 Chloramphenicol

Chloramphenicol stock solution contained; 30 mM chloramphenicol dissolved in methanol. A working concentration of 60 μM was used. Stock solution was stored at -20°C.

2.3.3 Kanamycin

Kanamycin stock solution contained 20 mM kanamycin. A working concentration of 60 μM was used. The solution was sterilised by passing through a 0.45 μm filter (Gelman) and stored at -20°C.

2.3.4 Tetracycline

Tetracycline stock solution contained 20 mM tetracycline in methanol. A working concentration of 20 μM was used. The stock solution was stored at -20°C.

2.3.5 Phleomycin

Phleomycin stock solution contained 250 mg/mL phleomycin (Cayla, Toulouse) in water. A working concentration of 50 mg/L was used.

2.3.6 Hygromycin

Hygromycin stock solution contained 50 mg/mL hygromycin (Roche, Mannheim). A working concentration of 200 mg/L was used in media.

2.3.7 IPTG

Isopropyl-beta-D-thiogalactopyranoside (IPTG) stock solution contained 0.84 M IPTG. The solution was sterilised by passing through a 0.25 μm filter (Millipore) and stored at -20°C.

2.3.8 X-Gal

5-Bromo-4-chloro-3-indolyl-beta-D-galactoside (X-Gal) solution contained; 50 mM X-Gal dissolved in dimethyl-formamide. The stock solution was stored at -20°C.

2.3.9 Complete supplement (CS).

Complete supplement (CS) contained; 20 g/L bacto-casamino acids, 20 g/L bacto-peptone, 20 g/L bacto-yeast extract, 3 g/L adenine, 0.002 g/L biotin, 0.002 g/L nicotinic acid, 0.02 g/L p-aminobenzoic acid, 0.002 g/L pyridoxine, 0.02 g/L thiamine, solution was filter sterilised with 0.45 μm Millipore filter unit. Stored at 4ºC

2.3.10 100 x trace solution.

100 x trace solution contained; 50 g/L KCl, 50 g/L $MgSO_4.7H_2O$, 1 g/L $ZnSO_4.7H_2O$, 1 g/L $FeSO_4.7H_2O$, 0.25 g/L $CuSO_4.5H_2O$. Solution was stored at 4ºC.

2.3.11 Centrifuged V8 juice.

Campbell's V8 juice was centrifuged at 5000 **g** for 10 minutes. The supernatant was decanted and used in media. Centrifuged V8 juice was stored at -20ºC.

2.4 *S. nodorum* strain

S. nodorum SN15 was supplied by the Department of Agriculture of Western Australia.

2.5 Wheat cultivar and growth conditions

The susceptible cultivar of wheat, Amery was supplied by the Department of Agriculture Western Australia. Wheat plants were grown in 10 cm pots filled with a perlite base (P500) covered by a main fill of vermiculite (G2) (The perlite and vermiculite factory, WA, Australia). Each pot was typically seeded with 10 seeds, and incubated at 20ºC in a 12 hour day-night cycle with Phillips grow-lux lighting. 2-week old plants were used for both whole-plant spray assays and detatched leaf assays.

2.6 *S. nodorum* culture growth conditions:

S. nodorum was routinely grown at 20ºC. Liquid cultures were 100 mL of media in a 250 mL flask. Cultures were shaken at 130 rpm in constant darkness.

Solid media cultures were grown in alternating 12 hours darkness, 12 hours near-UV light with a maximum at 350 nm.

2.7 Transformation of *S. nodorum*

2.7.1 Solutions

2.7.1.1 Protoplast wash solution

Protoplast wash solution contained; 600 mM $MgSO_4$. Solution was sterilised by passage through a 0.25 µm filter.

2.7.1.2 Glucanex digestion solution

Glucanex digestion solution contained, 1.2 M $MgSO_4$, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 15 g/L Glucanex enzyme (Novazyme). pH 5.8. Solution was sterilised by passage through a 0.25 µm filter.

2.7.1.3 1 M Sorbitol solution.

1 M sorbitol solution contained, 1 M Sorbitol, 10 mM Tris-HCl pH 7.5. Solution was sterilised by passage through a 0.25 µm filter.

2.7.1.4 Protoplast overlay solution.

Protoplast overlay solution contained; 0.6 M Sorbitol, 10 mM Tris-HCl pH 7.5. Solution was sterilised by passage through a 0.25 µm filter.

2.7.1.5 STC solution.

STC solution contained; 1.2 M Sorbitol, 10 mM $CaCl_2$, 10 mM tris-HCl pH 7.5. Solution was sterilised by passage through a 0.25 μm filter.

2.7.1.6 PEG solution

PEG solution contained; 600 g/L Polyethyleneglycol-4000, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5. Solution was sterilised by passage through a 0.25 μm filter.

2.7.2 Procedures

All manipulations were performed under aseptic conditions. CZV8CS liquid medium (100 mL) was inoculated with $5x10^7$ *S. nodorum* pycnidiospores and incubated at 20 ºC overnight (approximately 20 hours) while being shaken at 130 rpm. The culture was centrifuged at 3000 *g*/10 min and the media removed by decanting. The mycelium was washed with 100 mL 0.6 M $MgSO_4$ and then resuspended in 20 mL of Glucanex digestion solution. The resuspended mycelia was incubated at 28 ºC in a sterile glass Petri dish for two hours.

Digested mycelia was added to a 50 mL centrifuge tube and 5 mL of Protoplast overlay solution carefully layered on top while maintaining a distinct interface between the two solutions. The layered mycelia was centrifuged at 4000 *g* for 15 min. After centrifugation protoplasts concentrate at the interface, they were gently aspirated with a P1000 pipette , washed in 1 M Sorbitol solution and resuspended in 1 mL STC buffer. The protoplasts were then counted and resuspended at a concentration of 5×10^8 protoplasts/mL in STC. To transform, 100 μL of the protoplast solution was added to 3-7 μg of DNA construct in STC buffer and incubated at room temperature for 15 min. A control transformation was also prepared where an equal volume of STC buffer was

used in place of the DNA construct. PEG solution was then serially added to the protoplast-DNA mix as 2 x 200 µL aliquots followed by one addition of 800 µL, ensuring full mixing occurred after each addition. The protoplast-DNA-PEG mixture was incubated at room temp for 15 min, subsequently resuspended in 25 mL of CZV8 top agar (melted and cooled to 50ºC) and immediately plated out onto CZV8 protoplast medium (15 mL per plate). The control transformation mixture was resuspended in 10 mL CZV8 top agar. Five mL of protoplast-top-agar mix was added to each CZV8 protoplast plate. The inoculated plates were then wrapped in aluminium foil and incubated for 24 hours at 20ºC.

After the overnight incubation, 5 mL of CZV8 top agar (melted and cooled to 50ºC) containing 200 mg/mL phleomycin was added to each plate so that the entire surface was covered. The plates were then sealed and incubated at 20ºC in the dark until mycelial growth was observed on the surface of the agar. Individual colonies were isolated from the surface of the plate and placed onto a new CZV8CS-phleomycin plate.

2.7.3 PCR screening of *S. nodorum* transformants.

Purified genomic DNA (1 µL) was used as template for a PCR where primers that anneal outside DNA construct amplify the whole locus. The size of the amplicon revealed the nature of the integration event.

2.8 Southern blot

2.8.1 Digestion and separation of genomic DNA for Southern blot.

Genomic DNA (5 µg) was digested overnight at 37ºC, with 15 U of restriction enzyme, in a 300 uL reaction. Completed digests were concentrated using a Maxi Dry Lyo (Heto

Holten, Allerød, Denmark) vacuum concentrator to approximately 20-30 µL, before electrophoresis overnight at 30 V.

2.8.2 Transfer of nucleic acids to nylon membrane.

Electrophoresed nucleic acids were transferred from the gel to positively charged membrane (Hybond N+, GE Healthcare) by vacuum blotting using the VacuGene XL Vacuum Blotting Unit (GE healthcare). Vacuum blotting was performed according to the 'HCl-depurination and vacuum transfer of high molecular weight DNA' method supplied by the manufacturer.

2.8.3 Probe labeling.

Probe DNA was produced by PCR amplification of the region of interest from genomic DNA. The PCR amplicon was gel extracted prior to labelling. Probes were labelled with digoxygenin-dUTP (DIG) using the DIG High-prime random labelling system according to manufacturer's instructions (Roche applied science).

2.8.4 Hybridisation of probe to transferred nucleic acids.

Labelled probe was hybridised to transferred nucleic acids in DIG-easy-hyb solution (Roche applied science) at 42ºC overnight.

2.8.5 Detection of probe hybridised to transferred nucleic acids.

The method for probe labeling, hybridization and detection was as described in the Roche DIG Application Manual for Filter Hybridization (Roche applied science). The high stringency wash in 0.5 x SSC 0.1 % SDS was performed at 65ºC. All consumables for DIG detection were supplied by Roche applied sciences.

2.9 Plasmids

Table 2.1

Plasmids used in this study are listed here, vector maps are also shown in the appendix.

Plasmid name	Insert	Base vector	Size	Selection	Description	Reference
pGEM-T-easy			3000 bp	Amp	TA cloning vector	Promega
pAN8-1		pUC18	6089 bp	Amp, phleo	Fungal expression vector	GenBank accession: Z32751
pBluescript SK+			2958 bp	Amp	Cloning vector	Stratagene
pBSK-Phleo	Phleomycin cassette	pBluescript SK+	5231 bp	Amp, phleo	Phleomycin cassette ligated into pBluescript SK+.	(Solomon *et al.*, 2006d)
pGEM-TPS-5'	Tps1 5' flank	pGEM-T-easy	4044 bp	Amp	5' flank for pTPSKO	This study
pGEM-TPS3'	Tps1 3' flank	pGEM-T-easy	4107 bp	Amp	3' flank for pTPSKO	This study
pBSK-phleo-Tps3'	Tps1 3' flank	pBSK-phleo	6310 bp	Amp, phleo	Construction of pTPSKO	This study
pTPSKO	TPS 5' and 3' flanks	pBSK-phleo	7339 bp	Amp, phleo	Gene replacement vector, *Tps1*.	This study
pTripleX2			3589 bp	Amp	Base vector for *in planta* cDNA library.	Clontech
pSPORT			4109 bp	Amp	Base vector for *in vitro* cDNA library.	Invitrogen

2.10 Polymerase chain reaction (PCR).

2.10.1 PCR reagents

All PCR reagents were made in sterile H_2O unless otherwise mentioned.

2.10.2 DNA polymerase

Taq DNA polymerase (Fisher Biotech) was used in standard PCR reactions.

2.10.3 dNTP solution

dNTP solution contained 1.25 mM dATP.Li salt, 1.25 mM dCTP.Li salt, 1.25 mM dGTP.Li salt, 1.25 mM dTTP.Li salt.

2.10.4 10 x PCR buffer

Taq DNA polymerase 10 x buffer (Fisher biotech) contained 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 500 mM KCl.

2.10.5 Primer stocks

Primer solutions were diluted to a stock concentration of 100 μM in sterile H_2O. Primers used are listed in Table 2.2.

2.10.6 Standard PCR reaction components

Reactions contained; 2.5 μL 10 x PCR buffer, 1.0 μL dNTP solution, 0.5 μL of each primer, 1 U DNA polymerase, 15.4 μL sterile H_2O and 5 μL DNA template.

2.10.7 High-fidelity PCR reaction components

If an amplicon was to be used for transformation of *S. nodorum*, a high fidelity DNA polymerase was used. Reactions (20 μL) contained; 2.0 μL 10 x PCR buffer, 0.8 μL

dNTP solution, 0.5 μL of each primer (10 pmol/μL), 2 U DyNAzyme EXT DNA polymerase (Finnzymes), 11 μL sterile H_2O and 5 μL DNA template.

2.10.8 Thermal cycler settings

PCR reactions (25 μL) were incubated in 0.2 mL PCR tubes using a Perkin-Elmer 9600, thermocycler. The standard program used was; 95ºC for 2 minutes, 35 cycles of (95ºC 30 seconds, 60ºC for 30 seconds, 72ºC for 1 minute/kb amplicon), 72ºC for 5 minutes, 10ºC hold.

2.11 PCR Primers

Table 2.2
PCR primers used in this study

Name	Sequence 5'-3' (1)	Annealing temperature	Amplicon size	Note
AbdScrnF	TGTGGAACGCTTGCATAATGA	57ºC	WT 3028 bp, Mut 4495 bp	PCR screen *Abd1* KOs
AbdScrnR	AAAAACCGGCTTCACGAAGA			
AbdKO5'F	AATGACTCTTTTCCATCGGC	62ºC	937 bp	*Abd1* Overlap PCR construct
AbdKO5'R	TGTGACTTTTGGTTACGCCGTCTTCTCCACAACCTCCAGACTC			
AbdKO3'F	TCTCCTATGAGTCGTTTACCCAGAACGGCGTTATTGAGCTGAGT	62ºC	820 bp	*Abd1* Overlap PCR construct
AbdKO3'R	ACATGTTGAAGGGTCTGCC			
pAN8f	AGACGGCGTAACCAAAAGTCACA	60ºC	2424 bp	Phleomycin cassette
pAN8r	TTCTGGGTAAACGACTCATAGGAGA			
pBSK-phleoF	TTCGTTGACCTAGCTGATTCTGG	57ºC	2252 bp	Phleomycin cassette
pBSK-phleoR	CTCTTCGCTATTACGCCAGCTG			

Name	Sequence 5'-3' [1]	Annealing temperature	Amplicon size	Note
TPSKOFwd	AATATCATTCTTTCGTCACCCG	57ºC	WT 2151 bp	PCR screen *Tps1* KOs
TPSKORev	CATCTAGCCAAGCAGAACCA		KO 2639 bp	
TPS-probe3'	AAGCCATTGGGCGTTGTT	55ºC	254 bp with TPSKO3'F	*Tps1* Southern blot probe
TPSKO5'F	CTCGAGAGATCTAATAGATGCCATAA	55ºC	1044 bp	*Xho*I site
TPSKO5'R	AAGCTTTGTCATGTTTGCGGTATATA			*Hin*dIII site
TPSKO3'F	CTGCAGAGCAGCTCCCTTGCCGTTCT	56ºC	1107 bp	*Pst*I site
TPSKO3'R	GCGGCCGCTCTATAGATGGTGTACAGTC			*Not*I site
SNU00557-Fwd	CAACTGTCCGGAGCATCCCA	57ºC	154 bp	qPCR
SNU00557-Rev	AGTTTGCGTTGGAGGGGTCG			
SNU03877-Fwd	ATGCCGTCCACCAGAAGCAAGAA	57ºC	176 bp	qPCR
SNU03877-Rev	CGCGGAGCGTATGTGGCAAGA			
SNU08496-Fwd	ATCAAGGAGGGTGCCACGTTC	57ºC	196 bp	qPCR
SNU08496-Rev	AGGGAGCAGTTTCGGGTTCG			

Name	Sequence 5'-3' (1)	Annealing temperature	Amplicon size	Note	
SNU11873-Fwd	CGACATCCTCGGCCTCGAAT	57ºC	195 bp	qPCR	
SNU11873-Rev	ACAATGCCATGCATCCACGTC				
SNU12085-Fwd	GGTTGGGGAAAGCGGGAGTT	57ºC	169 bp	qPCR	
SNU12085-Rev	CGTGCTGAGCACCATCAACGT				
SNU12441-Fwd	ACAAGTCGGACGTCATTGCGA	57ºC	153 bp	qPCR	
SNU12441-Rev	TGTTCTCGAAGCTGGCTGGG				
SNU14274-Fwd	CACGAATCCGCTGGCACAGT	57ºC	169 bp	qPCR	
SNU14274-Rev	TGACGGGTGGTGTTGAGAGGA				
SNU16499-Fwd	GCGCATGGCTGCCAACTATG	57ºC	161 bp	qPCR	
SNU16499-Rev	TGGCGTTCAAGCTCTGCATCA				
ActinqPCR-F	AGTCGAAGCGTGGTATCCT	57ºC	165 bp	qPCR	SNOG_01139
ActinqPCR-R	ACTTGGGGTTGATGGGAG				

2.12 Restriction enzyme digestion of DNA

Restriction enzyme digestion was performed in a 25-75 μL total reaction volume. Reactions contained; DNA to be digested, 2-5 units of restriction enzyme, appropriate commercial enzyme buffer and sterile H_2O to the desired total volume. Reactions were incubated at 37°C for 1 hour or until digestion was complete as determined by agarose gel electrophoresis. Reactions were halted by incubation at 65°C for 10 minutes or by the addition of one quarter volume of sodium dodecyl sulphate (SDS) electrophoresis loading dye.

2.13 DNA ligation

A DNA ligation reaction (19 μL) contained 2 μL of 10 x ligation buffer (New England Biolabs), insert DNA, 20 ng of vector and sterile H_2O. DNA ligase (40 Units) (New England Biolabs) in 1 mL was added to the reaction, and incubated at 4°C overnight.

2.14 Escherichia coli strains.

XL1-Blue

XL1-blue was supplied by Clontech. Genotype was: endA1, gyrA96, hsdR17, lac –, recA1, relA1, supE44, thi-1, [F' lacI qZ ΔM15, proAB, Tn10] Note: Tn10 confers resistance to tetracycline.

BM25.8

BM25.8 was supplied by Clontech. Genotype was: supE44, thi Δ(lac–proAB) [F' traD36, proAB +, lacIqZ ΔM15] λimm434 (kan R)P1 (camR) hsdR (r k12– mk12–)

2.15 Bacterial transformation

E. coli competent cells were thawed on ice before use. The Gene Pulsar and Pulse controller apparatus (BioRad) were set to deliver 25 μF at 2.5 kV and 200 Ω. DNA (10 ng) was added to 40 μL of competent cells, mixed and incubated on ice for at least one minute. The cell suspension was added to a cold 0.2 cm electroporation cuvette (Invitrogen) and electroporated once at the above settings. Immediately after electroporation 500 μL of LB medium was rapidly added to the cells and incubated at 37°C for 30-60 minutes. After incubation the culture was plated on LB agar containing antibiotic selection and incubated at 37°C overnight. Blue white selection was used if supported by the transformed plasmid.

2.15.1 Blue/white selection

40 μL of X-Gal stock solution and 40 μL of IPTG stock solution were spread on the surface of solid-media plates.

2.15.2 *E. coli* competent cells preparation.

Two cultures of *E. coli* were grown overnight in 5 mL of LB medium at 37°C with antibiotic selection. The two overnight cultures were each added to a 2 litre flask containing 1 litre of LB broth and antibiotic selection. The culture was incubated at 37°C (300 rpm) for 3 hours until an A_{600} culture density of 0.5-0.6 absorbance units was reached. The culture was chilled on ice for 20 minutes and centrifuged at 4000 x **g** for 10 minutes to harvest the cells. The supernatant was discarded and the cells resuspended in 1 litre of H_2O (4°C). The cells were washed three more times, and were resuspended in 500 mL H_2O (4°C), 20 mL 10% (v/v) glycerol (4°C) and 4 mL 10%

(v/v) glycerol (4°C), respectively. The cells were then divided into 40 μL aliquots and stored at -80°C.

2.15.3 Plasmid DNA isolation.

Plasmid DNA was isolated using the Quantum prep plasmid purification kit, mini-prep size, according to the manufacturer's instructions (BioRad).

2.15.4 Genomic DNA isolation.

Genomic DNA was isolated from fungal tissue using the BioSprint15 plant kit (cat# 941517) and the BioSprint15 automated workstation (cat# 9000850) (Qiagen). Tissue was snap frozen and ground under liquid nitrogen prior to DNA isolation.

2.16 Agarose gel electrophoresis

2.16.1 Agarose gel electrophoresis method.

Agarose gels were made with 0.7-2.0 % (w/v) agarose (BioRad) dissolved in 1xTAE buffer (20 mM Tris, 10 mM glacial acetic acid, 1 mM Na_2EDTA (pH 8.5)). Electrophoresis was performed in 1 x TAE buffer at a constant 70-100 V. DNA fragments were visualised by soaking the gel in 5 μM ethidium bromide for 10-20 minutes, prior to exposure to shortwave UV light. Fluorescence was recorded by a BioRad 'gel-doc' system. Sizes of DNA fragments were estimated by comparing their migration to that of DNA size markers (1kb ladder, Promega).

2.16.2 DNA cleanup

2.16.2.1 Gel extraction.

DNA fragments were electrophoresed as described above before being excised from the gel and processed with the Qiagen gel extraction kit (Qiagen).

2.16.2.2 PCR product clean up.

PCR products that were present as single amplicons were cleaned using the QiaQuick PCR purification kit (Qiagen).

2.16.3 Automated sequencing of DNA.

The Sanger dideoxy chain termination method for sequencing reactions (Sanger *et al.*, 1977) was carried out using ABI Prism Big Dye 3.1™ chemistry (Applied Biosystems) and analysed by the SABC sequencing service on an ABI-3730 DNA sequencer (Applied Biosystems). Sequence data was viewed Vector NTI 10 (Invitrogen) and Finch TV software (Geospiza).

A subset of 384 sequencing runs were performed by AgGenomics (Bundoora, Australia). AgGenomics were supplied with plasmid clones as bacterial glycerol stocks, which they amplified by the TempliPhi method (GE biosciences) and sequenced.

2.17*S. nodorum* spore harvest

S. nodorum cultures (3 weeks old) grown on solid media were flooded with sterile water and scraped with a pipette tip to disturb the mycelial mat., then incubated at room temp for 5 minutes. The plate was re-scraped and the surface liquid aspirated from the plate, and filtered though a sterilised glass-wool syringe (5 mL syringe packed with 1 mL

glass wool). The spore solution was centrifuged at 4000 ***g*** for 10 minutes, the supernatant discarded and the spores resuspended in 1 mL sterile water. Spore numbers were counted by visualisation of the spore solution on a haemocytometer (Neubauer improved bright line).

2.18*S. nodorum* pathogenicity assays.

2.18.1 Growth of wheat cv. Amery.

Wheat seeds cv. Amery were surface sterilised for 5 min in seed sterilisation solution (1 % bleach (w/v), 5 % Ethanol) prior to sowing. Seeds were planted in 8 cm pots containing a base layer of perlite (The perlite and vermiculite factory, Jandakot) and the remainder filled with vermiculite (The perlite and vermiculite factory, Jandakot). Plants were grown at 22ºC in a 12 hour light-dark cycle.

2.18.2 Detached leaf assay (DLA).

Wheat plants cv. Amery were grown from seed for 2 weeks before the first primary leaf was detached, trimmed to 4 cm in length and mounted adaxial-side up on a square benzimidazole agar plate. A 5 uL aliquot of *S. nodorum* pycnidiospore solution ($1x10^6$ spores/mL in 0.01% (v/v) Tween 20)) was deposited on the centre of each leaf. The plate was sealed and incubated at 23ºC in a 12 hour light-dark cycle. Five leaves were prepared per strain tested. The infection was allowed to progress for 7 days until disease progression was documented. Lesion size was measured and numbers of pycnidia counted.

2.18.3 Whole plant spray (WPA).

Eight pots, each containing eight wheat plants cv. Amery were grown from seed for 2 weeks before inoculation with 12 mL of pycnidiospore solution (1×10^6 spores/mL in

0.01% Tween 20). The pycnidiospore solution was sprayed over the surface of the eight pots of plants using a pressurised air sprayer. Inoculated plants were covered and sealed to increase humidity, for a period of two days. After uncovering, disease was allowed to progress until 7 dpi, when the plants were scored for disease progression.

2.18.4 Whole plant spray disease progression scoring system.

After completion of the whole plant spray, plants were scored for disease symptoms. The scale used was from 1 to 10, with 1 representing a disease free plant and 10 being one that was completely dead (Solomon *et al.*, 2004a) .

2.18.5 Latent period assay (LPA).

Infected leaves from completed whole plant spray assays were detached, the top 2 cm removed, and trimmed to 4 cm in length and mounted on square benzimidazole agar plates. The plates were sealed and incubated at 23ºC in a 12 hour light/dark cycle. Pycnidia formation was monitored each day. The latent period was defined as the time taken until 50 or more stage 3-4 pycnidia were observed on each leaf (2.18.6). A minimum of five infected leaves were observed per strain.

2.18.6 Pycnidium growth stages

During the latent period assay and the detached leaf assay, pycnidia were scored by counting those that had reached certain developmental points, or stages. The stages were defined as published by Solomon *et al.* (2006d) and are summarised here. Stage one, round black pycnidium; stage two, swollen pink/black pycnidium; stage three, pink/black pycnidium with conical projection; stage four, pink/black pycnidium with a round ball of pink exudate (cirrhus) visible from ostiole; stage five, pink/ black pycnidium with cirrhus spreading over leaf surface.

2.19 Statistical tests

Two statistical tests were used to determine the significance of differences between mean abundances of metabolites. The t-test and the Tukey-Kramer significance test. Although the t-test is the most commonly used statistical test, it is limited to comparison of only two treatments per analysis. The Tukey-Kramer method is a more appropriate method when all comparisons are of interest and gives a more conservative likelihood of mean difference.

2.19.1 The t-test

The t-test was performed using Microsoft Excel software. The t-test was used to compare two mean values and calculate the probability (P value) that the two population means are the same.

2.19.2 Tukey-Kramer significance test.

The Tukey-Kramer test was performed using SAS JMP in 5.1 software, with an alpha level of 0.05 or 0.01, as noted. The Tukey-Kramer test can be used to assign levels to each mean, where means that share a common level are not significantly different. For example if the means of three treatments were all different they could be assigned levels of A, B, C for treatments one, two and three, respectively. This would also indicate that treatment one had the highest mean and treatment 3 had the lowest mean. Similarly if the three treatments were given levels of A, AB, B for treatments one, two and three, respectively, it would mean indicate that treatment one had the largest mean and was significantly different to treatment three and the treatment two was not significantly different to either treatment one or three.

2.20*S. nodorum* genome sequence access

2.20.1 Genomic sequence

Genomic contigs, auto-called genes and deduced protein sequences were retrieved from the Broad institute website (www.broad.mit.edu). Auto called gene and protein sequences have recently been deposited in the Genbank database, and now may be accessed there as well.

2.20.2 Expressed sequence tags (ESTs)

EST sequences were accessed via the NCBI Trace archive (www.ncbi.nlm.nih.gov/traces/trace.cgi?). Use the search terms:

CENTER_NAME = WIBR (needed for both libraries) and

SEQ_LIB_ID = G830K1 (for the *in planta* library) or

SEQ_LIB_ID = G831K1 (for the *in vitro* library)

or using a trace name identifier, for example

TRACE_NAME = 'G831P38FN20.T0'

Additional EST sequences can be accessed via the NCBI dbEST database. To retrieve all ACNFP submitted ESTs use the search term:

Phaeosphaeria nodorum Richard Oliver

Three libraries are available, named: "*S. nodorum* oleate-induced cDNAs"; "cDNA library of sporulating *Phaeosphaeria nodorum* SN15 on Wheat cv. Amery"; and "cDNA library of *Phaeosphaeria nodorum* grown on wheat cell walls".

Chapter 3

Cytology of sporulation in *Stagonospora nodorum*.

3.1 Introduction

Three notable studies describing asexual development of *S. nodorum* have been reported previously. Two reporting pycnidia development in culture and a third documenting the disease cycle and sporulation *in planta* (Douaiher and Halama, 2002; Douaiher *et al.*, 2004; Solomon *et al.*, 2006f). Douaiher (2004) prepared a superb account with ten detailed drawings of pycnidial development in culture, defining three major stages of asexual development; stage one: The pycnidial primordium; stage two: The formation and the extension of the pycnidial cavity and conidiogenesis; and stage three: The opening of the ostiole. The following description is adapted from Douaiher *et al.* (2004).

Pycnidial primordium are thought to arise when hyphal branches congregate at a single point and begin to entangle, forming the observed "mycelial knots" or "nodules". The peripheral cells proliferate by division, and produce a dense, fertile centre within the primordium. The fertile centre expands from around the periphery and begins to produce the conidiogenous cells. The structure increases in size and begins to develop a conical projection, the future ostiole.

The cells in the centre of the pycnidium begin to separate from each other, forming the central cavity. The peripheral cells become flattened and form the thin wall around the pycnidium. The apical cone becomes darker, and is also composed of flattened hyphal cells. Conidiogenous cells cover the interior surface, pointing toward the pycnidial cavity, and produce uninucleate pycnidiospores at their tips. These pre-spore cells form a dividing septum between themselves and the parental cell, and then elongate in the

direction of the ostiole, before detaching. The mature spores continue to fill the interior cavity as the pycnidium develops.

The opening of the ostiole releases the mature spores from the pycnidium. At this stage the pycnidium has effectively stopped developing new tissue types. The ostiole develops from the apical cone. It has been suggested that the terminal cells break apart under the increasing pressure from the build up of spores internally, producing the required opening. At this stage the pycnidia releases mature pycnidiospores onto the surface, as a pink cirrhus, containing spores in a mucilaginous goo.

The study of sporulation in *S. nodorum* required the various stages of asexual development to be characterised in the systems to be sampled for genomic and metabolomic studies. Once these stages were identified, the best time points for sampling would be established. While reports of *S. nodorum* pycnidia development have been made, none have yet described the early stages of pre-pycnidial development during an *in planta* infection.

3.2 Materials and methods.

3.2.1 Trypan blue staining

3.2.1.1 Trypan blue staining solution

Trypan blue staining solution contained 10 mL lactic acid, 10 g phenol, 16 mL 60% (v/v) glycerol, 4 mL water, 10 mg Trypan blue (Sigma).

3.2.1.2 Trypan blue staining procedure

Tissue was immersed in equal volumes of 100% ethanol and trypan blue staining solution and heated in a boiling water bath for 5 min, then incubated at room temp overnight. Staining solution was removed and the tissue destained in a saturated solution of chloral hydrate, prior to viewing.

3.2.2 Sectioning of infected leaves.

Entire leaves previously stained in trypan blue were sectioned using a hand sectioning technique. Briefly, leaves were inserted into a slit in a carrot support (Woolworths) and held while the entire support and leaf was thinly sectioned with a hand held double-edged razor blade (Wilkinson sword). Sections were floated on water to dissociate support from the leaf section, prior to mounting and viewing. the procedure was based on the method of O'Brien and McCully (1981).

3.3 Results

3.3.1 Infection progress from penetration to sporulation.

Asexual sporulation is a critical part of the lifecycle of *S. nodorum*. Spore development was carefully documented from *in planta* infections to determine the correct time points for transcript and metabolite sampling. The system used was a latent period assay. Disease progression of *S. nodorum* SN15 on wheat cv. Amery was monitored each day until mature pycnidia were seen. Fungal structures within infected leaves were stained with trypan blue and viewed under light microscopy.

The initial stage of infection (7 dpi) involves spores germinating on the leaf surface, hyphae growing over the surface, and hyphae penetrating the leaf surface. Entry is possible in several ways, via stomata openings, around the base of a trichome or directly through the cuticle (Solomon *et al.*, 2006f). Figure 3.1 A shows a composite image taken of a site of early infection. The hyphae have generally tracked toward stomata and have made direct contact eight times with the twelve visible stomata. The preference for stomata is more clearly shown in Figure 3.1 B, where the location of stomata are highlighted in green and the hyphae are overlaid with a black line. Two instances where hyphae have made direct contact with the stomata are shown at higher magnification in Figure 3.1 C and D. An example of successful stomatal entry was observed at 7 dpi (Figure 3.1 E), although clear examples of stomatal penetration such as this were rarely observed.

Once the fungus had traversed the leaf surface, the adjacent plant epidermal and mesophyll cells became brown and withered, although the structure of the vascular

Figure 3.1

bundle remained largely intact throughout the infection. Figure 3.2 A and B show regions of the leaf at 10 dpi where the epidermis had begun to break down during the early stages of lesion development; damaged plant cells stained light blue within the lesion. At this stage, the leaf was largely free of infection and the fungus had very little macroscopic presence. One day later, at 11 dpi, the lesion became clearly outlined and contained necrotic cells (Figure 3.2 C). The lesion was defined by brown coloured tissue and disrupted epidermal and mesophyll cell structure. Chlorosis continued well beyond the initial site of infection (data not shown). By this time, fungal hyphae had begun to spread rapidly and invaded the collapsed mesophyll cells while also continuing to colonise the leaf surface (Figure 3.2 D). The surface hyphae branched regularly as they grew.

Once significant plant cellular breakdown had occurred (Figure 3.3 A), the infection progressed more rapidly. Chlorosis of the leaf occurred more quickly if multiple lesions were present on the surface of the leaf. Over the course of another 2-5 days the entire leaf began to become chlorotic and the hyphae ramified and spread quickly throughout the interior of the leaf (Figure 3.3 B). The initial site of infection contained the most damaged cell structures, while outside of that area, the plant cells were chlorotic but did not lose their shape or become brown in colour. When hyphae colonised these outer regions, their growth inside the leaf appeared unhindered and uniform (Figure 3.3 C). Asexual development began once the fungus had significant access to the interior of the host. Although the hyphal front had spread far beyond the initial site of penetration, the first pycnidia formed just around that area (Figure 3.3 D and E). Mature or semi-mature pycnidia were observed as roughly sperical, densely-stained structures positioned around the periphery of the initial lesion.

Figure 3.2

Figure 3.3

3.3.2 Specific stages of pycnidial development

As asexual sporulation is the focus of this study, a more detailed analysis of pycnidial development was performed. The infection process across a single leaf was not completely uniform in terms of pycnidial development. Once the infection has consumed a large area of the leaf, many different stages of pycnidial development were observed at the same time. At late-infection stages, the region immediately surrounding the initial site of infection contained the most advanced stages of pycnidia development, while further away, many of the primordial states were seen. Although there was a general progression of developmental stages across the leaf, individual examples of most stages could be seen in all areas. These developmental stages are shown in Figures 3.4, 3.5.

The earliest stage of pycnidial development observed is shown in Figure 3.4 A and B. These were very simple hyphal aggregations correctly termed "mycelial knots", with a diameter of 15 µm (Figure 3.4 A). The aggregation grew larger to 25–30 µm without appearing to change the shape or overall organisation (Figure 3.4 C, D). The hyphae connecting the knot to the rest of the colony appeared thicker than surface hyphae (Figure 3.4 C). Once a structure of around 30 µm was formed, it became a denser aggregation called a "pycnidial primordium" as shown in Figure 3.4 E, F. As the pycnidial primordium developed further it became a more spherical shape with a defined boundary (Figure 3.5 A, B). The next stage of development observed was the "immature pycnidium" shown in Figure 3.5 C and D. A transverse section of the immature pycnidium showed that it was a densely packed, uniform structure with a very clear margin (Figure 3.5 C). In this case the pycnidium was developing amongst the mesophyll cells, just beneath the layer of epithelial cells. This image also showed how

Figure 3.4

Figure 3.5

the central vascular bundle of the leaf was not invaded by hyphae and maintained its cellular structure even at a late stage of the infection cycle.

As the pycnidium approached maturity the interior differentiated into pycnidiogenous tissue. It then produced large amounts of pycnidiospores suspended in a thick cirrhus. Mature pycnidia are shown in Figure 3.5 E and F, respectively. The pycnidia shown here were about 100-150 μm in size. In Figure 3.5 E, the pycnidium has ruptured during sample preparation, and the released pycnidiospores are visible on the surface of the leaf. Once mature, the pycnidium forms an ostiole that protrudes above the surface of the leaf. Two mature pycnidia can be seen releasing pycnidiospores from their ostioles (Figure 3.5 F). The pycnidium was a brown colour and the ostiole visible as a conical protuberance that was a shade darker. The cirrus was orange-pink in colour, and was observed to spread onto the surface of the leaf without any prior disturbance.

Once the full asexual cycle had been documented during a latent period assay, appropriate time-points for sampling tissue with the early sporulation structures could be chosen.

3.4 Discussion

The growth of *S. nodorum* SN15 on wheat during a latent period assay was documented in order to define the correct time points for transcript and metabolite sampling. The overall behaviour of *S. nodorum* infection cycle has already been documented in the literature

3.4.1 Sampling for transcript profiling and metabolite analysis.

Excised lesion tissue would be suitable at 14 dpi for *in planta* transcript profiling capturing fungal mRNAs, while samples taken after 8 dpi, 10 dpi and 12 dpi were used for sampling metabolites. A late stage of infection was chosen for cDNA library construction as the amount of plant transcripts would need to be minimised. At 14 dpi most of the lesions were large enough to be easily excised and necrosis was abundant, reducing unwanted transcripts. In addition, many different stages of pycnidial development would be present, increasing the diversity of the cDNA library. Samples taken at 8dpi, 10 dpi and 12 dpi cover non-sporulating to sporulating situations for metabolite analysis. At 8 dpi, only small lesions were observed, with few if any sporulation structures. 10 dpi was an intermediate stage, where it was expected there would be some pre-pycnidial structures present. By 12 dpi the leaf would be mostly dead and many different pycnidial stages would be present.

3.4.2 The infection cycle of *S. nodorum in planta.*

This study largely supports the observations regarding the infection cycle reported previously. One point of contrast was that penetration was rarely observed to occur via stomata, while it was previously reported as occurring approximately equally between stomatal and non-stomatal methods (Solomon *et al.*, 2006f). The current study did not

sample a large number of penetration attempts and as such is likely to be skewed by natural variation within a small sample. Interestingly, although few successful stomatal penetrations were observed, taxis toward stomata was seen in one instance. This has not been reported for *S. nodorum* previously, and would be make for interesting further study to confirm the observation. It may be that *S. nodorum* is more attracted to leaf openings under different environmental conditions. The infections here were latent period assays, where entire 2-week-old plants were sprayed with pycnidiospores and kept in continuous darkness for 48 hours before exposure to a 12 hour light-dark cycle. In Solomon *et al.* (2006f), detached leaf assays were used, which involve infection of a cut leaf, mounted on agar and growth is always in a 12 hour light-dark cycle. Perhaps these two assays modify the stomatal behaviour (perhaps either opening frequencies or gas exchange rates) of the wheat leaf differently and thus the behaviour of the pathogen.

3.4.3 Asexual development of *S. nodorum in planta*.

The observed asexual development structure were all in accordance with those reported by Douaiher *et al*. (2004) during growth in culture. This fits with the profile of a fungal necrotroph. Absorbing nutrient from dead plant tissue means that there is no requirement for specialised feeding structures during growth *in planta*, such as those seen in biotrophs. Once host defence has been overcome, growth continues without any apparent inhibition in a very similar fashion to that observed in culture.

Light microscopy of Trypan blue stained samples was sufficient to observe all of the stages, although it was difficult to find an appropriate focal plane for most specimens. This is due to the adaxial surface of the wheat leaf containing large variations in height depending on vein position. An interesting thought suggests trying infection on the underside of the leaf. The abaxial surface still contains stomata, yet is not crevassed

like the upper surface. This could present the fungal structures in an even plane of focus, allowing better visualisation of intact structures. Hand-sectioning of pre-stained samples also provided an unexpectedly high level of detail, without requiring fixation or embedding of the tissue.

Chapter 4

Transcript analysis of *S. nodorum* growth *in planta* and *in vitro*.

4.1 Introduction

As part of a multi-platform investigation to isolate sporulation specific genes, transcript analysis was used to identify genes that were up-regulated with sporulation.

4.1.1 Transcriptomics.

The transcriptome describes the entire population of mRNAs or gene transcripts present in a given biological situation. Therefore, transcriptomics refers to the analysis of the transcriptome. While transcription does not necessarily correlate with enzyme activity or protein abundance, it is still a reliable indicator of changes in cellular processes. The study of gene expression at a genome level is generally performed using one of three main technologies, DNA microarray, serial analysis of gene expression (SAGE), or EST library analysis. Each technology has limitations and advantages.

4.1.1.1 DNA microarray.

As a refinement of colony-spotted nylon arrays of the 1980s, high-density DNA microarrays have rapidly developed during the genomics era ever since they were first reported in 1995 (Schena *et al.*, 1995; Stoughton, 2005). The technology involves a high-density array of DNAs on a solid support, which is hybridised with a labelled probe, derived from mRNA. The specific hybridisation between an mRNA derived probe and a defined sequence can be recorded as an analogue signal approximating the transcript abundance for a particular gene. Microarrays are the most rapid method to analyse transcription changes across an entire genome. Drawbacks include the expensive and specialised equipment and the requirement to have a defined set of probes to constitute the array, usually encompassing the entire set of genes from a

particular organism. In addition, a large number of replicates are needed in order to achieve a high degree of statistical power.

4.1.1.2 SAGE

SAGE is a gene expression profiling technique based on the capture and analysis of short sequences derived from mRNAs. The technique was developed in the same year as DNA microarrays (Velculescu *et al.*, 1995). SAGE produces sequence tags from mRNAs, which are concatemerized and cloned prior high-throughput sequencing. Sequence data is deconvoluted into individual tags and the frequency of occurrence, providing relative transcript abundance for each gene identified. The method is more economical and rapid than individual sequencing of entire cDNA clones. A genome sequence is not required, which reduces overall cost. Difficulties involve the potential ambiguity of short sequence tags and the high cost of sequencing consumables. Studies are usually statistically weak unless biological replication is included in the experimental design.

4.1.1.3 EST analysis

EST libraries have also proved a useful tool for gene expression analysis. In a similar principle to SAGE, mRNAs are cloned as cDNA libraries, ideally with one full-length transcript per clone. The library is sequenced, in a high-through-put manner with only one or two runs per clone. Each sequence is called an EST. The technique is similar to SAGE analysis, but fewer, larger transcripts are captured. Transcript frequency across different libraries can be used to infer gene expression changes for a large number of genes (Audic and Claverie, 1997). The main benefit is that cDNA libraries have many potential uses, including expression analysis, gene annotation and mutagenesis studies.

In particular, large EST datasets allow evidence-based annotation of new whole-genome sequences.

In this study, EST data derived from two cDNA libraries were used to infer gene expression differences between sporulating and non-sporulating cultures.

4.1.2 EST studies of filamentous fungi.

EST-based gene expression studies on filamentous fungi have become increasingly common. They are often performed as an initial foray into genomic research, providing an economic yet valuable resource for genomic research on organisms with limited resources.

EST libraries have been described in a broad range of filamentous fungi including: *Ustilago maydis* (Sacadura and Saville, 2003), *Mycospharella graminicola* (Keon *et al.*, 2005), *Fusarium verticillioides* (Brown *et al.*, 2005a) and *F. graminearum* (Trail *et al.*, 2003), *Aspergillus niger* (Semova *et al.*, 2006), *Beauveria bassiana* (Cho *et al.*, 2006), *Magnaporthe grisea* (Kim *et al.*, 2001; Soanes *et al.*, 2002), *Uromyces fabae* (Jakupovic *et al.*, 2006), *Blumeria graminis* (Eichmann *et al.*, 2006) and *Sclerotinia sclerotiorum* (Sexton *et al.*, 2006). Basic EST numbers and library details are listed in Table 4.1.

Comparative studies of EST libraries can be used to determine genes correlated with specific development conditions, for example; pathogenesis, toxin production, reproduction, or germination.

Table 4.1

(Ebbole *et al.*, 2004)

Sequencing of a cDNA library from *U. maydis* showed that identifiable transcripts within germinating teliospores most often classified mostly as belonging to protein biosynthesis and metabolism functions (Sacadura and Saville, 2003). The EST data also lead to the identification of a homologue of peredoxin which was confirmed to be upregulated in dormant spores. It was suggested that its transcript may be stored in spores to enable rapid protection of the nascent hyphae from oxidative damage.

A study of *M. graminicola* included three libraries; from budding conidia, pseudo-hyphal growth, and late-infection of the barley host (Keon *et al.*, 2005). Overall frequencies of cellular classifications did not greatly change between libraries, but there was some expansion of the groups 'protein synthesis' and 'protein fate' during growth *in planta*. This library was made from tissue containing asexual pycnidia, perhaps indicating that an increased rate of protein production was required for sporulation. While the functional classification groups did not change their abundance between libraries, most genes within those groups were not expressed in multiple libraries. It was suggested there was a highly flexible transcriptional system tailored to certain conditions. The study also revealed that heat shock chaperone proteins were over represented in the *in planta* library, perhaps an indication of the stressful environment of a late-stage infection.

Production of the potent mycotoxin fumonisin was studied in *Fusarium verticillioides* by analysis of a large EST dataset derived from cultures grown in toxin-inducing and non-inducing conditions (Brown *et al.*, 2005a). Nine genes were identified as potential fumonisin regulators, and a novel gene (*Fum20*) was identified within the fumonisin gene cluster. Alternate splicing of fumonisin gene cluster mRNAs was also detected.

This study alone demonstrates the broad applications of EST data in the study of physiological processes at the gene level.

Several *M. grisea* EST datasets have been analysed to identify pathogenicity-related genes (Soanes and Talbot, 2005). They identified that a hydrophobin (*Mpg1*), an arabinofuranosidase, a xylanase and a beta-glucosidase were upregulated during growth on rice cell walls. The highest upregulation was seen for formate dehydrogenase, which was thought to possibly indicate a metabolic shift in response to unfavourable conditions within the plant. Later, a comprehensive comparison was performed of ESTs deposited at the COGEME database (Soanes and Talbot, 2006). Over 57,000 ESTs, representing 13 different fungi and 2 oomycetes, were compared to seven fungal genomes including three pathogens and 2 saprophytes. They were surprised to find only 19 unisequences that were pathogen-specific. Among the 19 genes were two ion-transporting ATPases, a methyl transferase, and an aminotransferase.

One common theme among all published accounts of fungal EST collections is the abundance of transcripts with either no functional annotation or no match within gene databases. The proportion of unknown transcripts is often 30 - 50% of the entire population (Keon *et al.*, 2005; Sacadura and Saville, 2003; Soanes and Talbot, 2006). This huge collection of unidentified genes represents both a massive hurdle in gene discovery research and a potential treasure trove of novel functions.

EST libraries have been shown to be both important resources in genome annotation and powerful tools in comparative gene expression studies. In this study two libraries used to generate ESTs for the *Stagonospora nodorum* genome initiative were

recognised as an important resource for the study of sporulation in *S. nodorum* at the genomic level. Both sporulating and non-sporulating growth states were compared to identify genes upregulated during sporulation *in planta*.

4.2 Materials and methods

4.2.1 RNA isolation for *in planta* cDNA library construction.

RNA for the *in planta* cDNA library was extracted from pooled lesions excised from latent period assay leaves infected with SN15, harvested at 10, 11 and 12 dpi. Tissue was snap frozen and ground under liquid nitrogen. The TRIZOL (Invitrogen) method was used to extract total RNA from the ground tissue, according to manufacturer's instructions. RNA was resuspended in DEPC-treated water.

4.2.1.1 DEPC-treated H_2O

DEPC treated H_2O contained 0.6 mM diethylpyrocarbonate (DEPC). Solution was autoclaved prior to use.

4.2.2 DNAse treatment of *in planta* RNA.

Before use, RNA extracts were DNAse treated with DNA-free DNAse (Ambion) according to the manufacturer's instructions.

4.2.3 *In planta* cDNA library construction and manipulation.

The *in planta* cDNA library was constructed using the SMART cDNA library construction kit, catalogue# K1051-1 (Clontech). All manipulations were performed according to the manufacturer's instructions, including cDNA synthesis and ligationo f clones (www.clontech.com; manual PT-3000-1). Phage DNA was packaged using the GigapackIII Gold phage packaging system (Stratagene), according to the manufacturer's instructions. Phage were grown, amplified and mass-excised to bacterial clones according to the SMART cDNA library construction kit manual

protocols. The SMART cDNA library construction kit produced clones in the pTripleX2 bacterial vector.

4.2.4 *In vitro* oleate-induced cDNA library

The *in vitro* oleate-induced cDNA library was constructed by Rob Lee, ACNFP, Murdoch University and is used here with permission. As the methodology to construct the library has not been published elsewhere, it will be described below. Oleate was chosen as a carbon source to mimic the metabolic situation present during spore germination.

4.2.4.1 Growth conditions for *in vitro* oleate-induced cultures.

100 mL minimal medium + Sucrose (0.5% w/v) was inoculated with *S. nodorum* SN15 pycnidiospores (2.75×10^7) and incubated at 22 ºC with shaking (130 rpm) for 4 days. Mycelia was harvested by centrifugation at 2500 g/5 min, washed twice in sterile milli-Q water and added to 100 mL of minimal media 0.05 % (v/v) Tween 80 with 0.2 % (w/v) oleate as the sole carbon source. The culture was incubated as before for 30 hours before being harvested by centrifugation, washed in sterile water, snap frozen in liquid nitrogen and freeze dried in a Maxi Dry Lyo (Heto Holten, Allerød, Denmark).

4.2.4.2 cDNA library construction.

Messenger RNA was extracted from Oleate induced SN15 tissue using the Messagemaker mRNA purification-cloning kit (Gibco/Invitrogen) according to manufacturer's instructions. 2.3 µg of mRNA was used as template for reverse transcription to cDNA, prior to ligation into the pSPORT1 vector (Gibco/Invitrogen). The final library was estimated to contain 500,000 clones.

4.2.5 Bioinformatics.

4.2.5.1 Blast searches

Bulk BlastN, X and P searches were performed using Seqtools software (S. Rasmussen, www.seqtools.dk) (Altschul *et al.*, 1997). Individual searches were performed at the NCBI website www.ncbi.nlm.nih.gov.

4.2.5.2 EST trimming

ESTs were trimmed using the Seqtools program. Firstly the 5' region was trimmed. The first 100 bp of forward sequences and 150 bp of reverse sequences was removed, and then sequences were trimmed 3' of the first N at the 3' end. An iterative trimming procedure was used, which examined the last 100 bp at the 3' end and checked for similarity to the cloning vector using the BlastN algorithm. The iterative procedure was required because the software used for trimming was susceptible to incorrect removal of insert sequence if a larger region was allowed to be examined. Poly-A tail sequence was also removed. If a significant match was identified between the EST and the vector sequences, the region was removed. The *in planta* library was cloned in the vector pTriplEX2, the *in vitro* library was cloned in pSPORT1 vector. This procedure was repeated until matches to vector sequence were no longer found. At this point the ESTs were considered free of both vector and poor quality sequence.

4.2.5.3 EST clustering

ESTs were individually compared using the blastX algorithm to the deduced amino acid sequences for the Broad institute *S. nodorum* auto-called gene set. The auto-called gene list was used so that an EST would be correctly clustered, even if didn't overlap another EST. If an alignment had an expect score of $1x10^{-6}$ or less it was considered a significant match. The limit was set to allow short, nearly exact, matches to still be

assigned correctly. A less-stringent expect score limit also meat that incorrectly annotated genes would still be correctly matched to their ESTs. The best match for each EST was defined as the parent gene for that EST. For the sake of speed, ESTs that did not match a protein sequence were not included in the analysis. By tallying the numbers of ESTs matching each gene, transcription rates were inferred.

4.2.5.4 EST annotation.

The deduced protein sequence for each *S. nodorum* autocalled-gene was compared to the Genbank non-redundant database using the BlastP algorithm. The top twenty alignments were recorded. The match with the lowest expect score was recorded as the top blast match. If the top hit contained a non-informative description, e.g. "hypothetical protein" the list of descriptions was sequentially scanned for matches with a more descriptive annotation, the next match with a descriptive annotation was recorded as the "filtered best blast match". Genbank accession numbers of hits, expect scores and the length of the alignment were recorded.

4.2.6 Growth of SN15 *in vitro* for sporulating and non-sporulating cultures.

Cultures consisted of five minimal media agar plates per replicate, they were spread with SN15 spores (1 x 10^6 spores in 50 µL water) and incubated at 20ºC in the dark for 4 dpi and 18 dpi. Mycelium was scraped from the plate surface and immeadiately snap frozen in liquid nitrogen. Frozen mycelium was ground under liquid nitrogen and RNA extracted by the Trizol method according to manufacturer's instructions. After 4 dpi the colony was in early growth phase and no pycnidia had formed at all, the colony was not melanised. After 18 dpi the colony was mature, highly melanised and pycnidia were abundant.

4.2.7 Reverse transcription of RNA for quantitative PCR.

All solutions and equipment used during cDNA synthesis were free of RNase. Total RNA (1 μg) was reverse transcribed to cDNA using iScript reverse transcriptase premix according to the manufacturer's instructions (BioRad). The iScript premix produces a mixed population of random-primed and poly-A tail primed cDNAs. RNA from three biological replicates was pooled prior to cDNA synthesis. cDNAs were used as template in PCRs at 1:50 dilution for *in vitro* grown SN15 samples, and at 1:5 dilution for *in planta* grown SN15 samples. Genomic DNA contamination was monitored by the use of intron-spanning primers and observation of amplicaon size. No DNA contamination of RNA was observed in any experiment.

4.2.8 Quantitative PCR.

Quantitative PCR (qPCR) reactions consisted of 10 μL iQ SYBR green supermix (BioRad), 5 μL of a forward and reverse primer mix (each at 1.2 μM), and 5 μL of template cDNA or gDNA. Reactions were held in 20 μL tubes (Corbett) and incubated in a Rotor-gene 3000 thermocycler (Corbett research). Cycling conditions were, initial denaturation step for 3 min at 95ºC, then 35 cycles of 10 s 95ºC, 20 s 57 ºC, and 72 ºC 20 s. Completion of cycling was followed by collection of a melting-point curve, starting at 72ºC for 45 s, then ramping at 1ºC/5s to 95ºC. Fluorescence of SYBR green I was excited at 470 nm and collected at 510nm, with a gain setting of 5. Fluorescence from template of unknown concentration was compared to that from genomic DNA standards, of 25, 2.5, 0.25 and 0.025 ng/reaction. All reactions were performed in duplicate. Data was analysed using the Rotorgene software version 6.0 (Corbett research).

4.2.9 Overlap PCR for *Abd1* knockout construct.

Overlap PCR was used to join three amplicons in a single PCR reaction, for subsequent use as knockout construct. The *Abd1* gene referred to in the methods here is SNOG_14274.1, encoding a putative arabitol 4-dehydrogenase, as referred in this chapter.

4.2.9.1 DNA template

DNA template for fusion PCR consisted of the three amplicons (*Abd1* 5' flank, phleomycin cassette, and *Abd1* 3' flank), in an eqimolar mix (50 nM each). Abd1-5' flank (937 bp) was amplified by PCR from SN15 genomic DNA using primers AbdKO5'F and AbdKO5'R. Abd1-3'flank (820 bp) was amplified by PCR from SN15 genomic DNA using primers AbdKO3'F and AbdKO3'R. The phleomycin cassette (2424 bp) was amplified from pAN8-1 with primers pAN8F and pAN8R. The desired PCR amplicons were purified by gel extraction prior to use in fusion PCR.

4.2.9.2 Reaction components

Initially, trial fusion reactions were performed to check the performance of the construct design, and to optimise parameters for the cleanest amplicon generation. Trial fusions were performed for all constructs. Trial fusion reactions (20 µL) contained; 2.0 µL 10 x PCR buffer, 1.6 µL Takara dNTP solution (2.5 mM each), 0.2 µL of combined primer stock (5 µM of each primer), 2 U Takara EX Taq DNA polymerase (TaKaRa), 15.9 to 13 µL sterile H_2O and 0.1 to 3 µL DNA template. Template concentration in the trial fusion reaction was titrated to find the optimal amount, prior to bulk fusion (8 x 50µL reactions). Bulk fusion products were purified by gel extraction, which typically yielded 2-4 µg of construct, enough for a single transformation.

4.2.9.3 Thermocycler settings

The thermocycler program used for *Abd1-KO* overlap PCR was; 95ºC for 2 minutes, 35 cycles of (95ºC 15 s, 57ºC for 15 s, 72ºC for 4 min 30 s), 72ºC for 5 minutes, 10ºC hold).

4.3 Results

4.3.1 RNA isolation for cDNA library

In order to identify genes expressed during sporulation *in planta* a *S. nodorum* SN15 cDNA library was constructed. RNA for the library was isolated from *S. nodorum* lesions on wheat. A range of lesion sizes were harvested, covering early to late stage infections. The quality of the RNA was checked by electrophoresis of the RNA on a non-denaturing agarose gel. Two major bands corresponding to ribosomal RNAs were visible in the selected extracts, with minimal degradation evident (data not shown).

4.3.2 cDNA library construction

Total RNA was used as starting template for a cDNA library constructed using the CLONTECH Smart cDNA Library construction kit. The method was designed to enrich for full length cDNAs while avoiding reverse transcription of structural ribosomal RNAs. The unamplified phage library was estimated to contain 1 x 10^6 clones. The vector contained blue/white selection to screen for small or absent inserts. It showed that 92% of the clones in the library failed to express beta-galactosidase due to disruption of the cloning site (data not shown). An aliquot of the library containing 220,000 clones was amplified to create a high-titre stock for storage. A sample of the phage library (100,000 clones) was mass excised, producing the bacterial vector pTripleX2, suitable for a sequencing project. The resultant bacterial colonies, each derived from a single cDNA clone, were pooled and a mass plasmid DNA isolation performed. This plasmid pool (containing all cDNA clones) was then used to transform *E. coli* DH10B competent cells, a strain suitable for the production of plasmid for sequencing. Single ampicillin resistant colonies were individually isolated, cultured and

plasmid DNA extracted for sequencing. An initial batch of 60 clones were sequenced from the 5' end to check the insert size and redundancy. The results showed the redundancy was low at approximately 6%. In addition, 30 clones were digested with restriction enzymes *Eco*RI and *Xba*I to release the cloned insert and check the average size. Agarose gel electrophoresis of the products showed that the average insert size was 1.2 kb.

The library was deemed suitable for a larger sequencing effort. A further 384 clones were isolated and sequenced. The clones were sequenced from the 5' end of each clone using the pTripleX2 sequencing primer.

4.3.3 Analysis of the pilot study of the *in planta* library

The pilot study produced 444 ESTs. It showed the library was a useful representation of *S. nodorum* SN15 mRNA during growth *in planta*. Redundancy was low and wheat sequences accounted for only 12% of the library. A large-scale sequencing project was considered feasible. A further 5000 clones from the *in planta* library were sequenced at the Broad Institute as part of the *Stagonospora nodorum* genome sequencing project. (Broad Institute of Harvard and MIT, www.broad.mit.edu). In this project, paired 5' and 3' sequences were obtained from each cDNA clone. At the same time, 5000 clones from an *in vitro* oleate-grown SN15 cDNA library (courtesy Rob Lee, ACNFP, Murdoch University, Western Australia) were sequenced in both 5' and 3' directions. The two libraries represented examples of *in planta* and *in vitro* growth.

The 20,000 expressed sequence tags (ESTs) formed the basis of a bioinformatics-based strategy to identify genes up-regulated during growth *in planta* when compared to the *in vitro* culture.

4.3.4 Trimming and sequence quality control

The Broad institute released the expressed sequence tag (EST) data to the public as unprocessed sequencing chromatograms with associated FASTA files of the interpreted sequence. ESTs were trimmed to remove poor quality sequence and vector sequence.

Hsiang and Goodwin (2003) described a novel method for the resolution of plant and fungal sequences from a mixed cDNA library. They showed that comparison to a single plant and a single fungal genome by TBlastX was faster and more accurate than comparison to the Genbank nr database. Plant transcripts present in the *in planta* library were identified by comparison each EST to a plant genome (*Arabidopsis thaliana*) and to the *S. nodorum* genome using the TBlastX algorithm. The *A. thaliana* genome was used instead of a wheat EST collection as it was a smaller, more complete sequence, than wheat ESTs or the rice genome, and was able to be handled by the computer system used for the analysis. If the EST matched a plant sequence better than a *S. nodorum* sequence, it was discarded from the analysis. Remaining ESTs were clustered by BlastN alignment to the *S. nodorum* auto-annotated gene set released by the Broad institute. Each EST was assigned to the gene producing the best alignment.

Although each library initially produced around 10,000 sequences, after trimming and removal of plant ESTs had been performed, the numbers of ESTs per library had reduced significantly. The *in planta* ESTs contained 5515 sequences and the *in vitro*-grown ESTs contained 6713 sequences, making a total of 12228. Analysis of the length of the ESTs remaining in each dataset revealed the following statistics. The *in planta* ESTs had a median length of 510 bp, a maximum length of 866 bp and a minimum length of 58 bp (Figure 4.1).

Figure 4.1

The *in vitro* library contained ESTs with an median length of 579 bp, a maximum length of 977 bp and a minimum length of 71 bp. Both libraries appeared to have two populations within the distribution, as described by sequence length.

4.3.5 Clustering of ESTs.

Once plant ESTs had been removed, the EST population was aligned to the autocalled genes of *S. nodorum* genome sequence. In order to cluster ESTs derived from a single gene, each sequence was compared to the autocalled genes in the *S. nodorum* genome database (www.broad.mit.edu) using the BlastN algorithm. The ESTs grouped into 2252 unigenes composed of 1790 clusters with two or more ESTs, and 462 composed of single ESTs or singletons (Table 4.2). The *in planta* library contained more singletons than the *in vitro* library. The expressed unigenes identified were sorted according to presence in one or both of the two libraries. Of the 2252 identified unigenes, 1021 were only found in the *in planta* library, 804 were only found in the *in vitro* library, and only 427 unigenes were present in both libraries (Figure 4.2 A). The number of ESTs attributed to each unigenes was also counted, 2287 ESTs were only found in the *in planta* library, 5637 were found in both libraries and 3704 ESTs were only found in the *in vitro* library (Figure 4.2 B).

4.3.6 Gene annotation

The deduced aminoacid sequence for all genes with EST matches were checked for similarity to other protein sequences in the Genbank nr database, using the BlastP algorithm (Altschul *et al.*, 1997). The top 20 hits per gene were recorded. If the most similar sequence did not have an annotated role, the list was searched for the next best hit with an annotated function. Expect scores, descriptions and accession numbers of all hits were recorded.

Table 4.2

Figure 4.2

4.3.7 Gene expression analysis by EST abundance.

The transcript abundances from the *in planta* and *in vitro* libraries could be compared as they were not subtracted libraries and had an similar number of ESTs, providing a crude but useful indicator of gene expression differences between the two growth states. The probability that the two libraries contained identical EST abundances for a particular unigene was calculated based on the observed abundances. The method of Audic and Claverie was used to calculate a P value, which took into account the different sample sizes (Audic and Claverie, 1997). The EST cluster lists were sorted to reveal genes that were statistically verified to be represented more frequently *in planta* than *in vitro* and *vice versa* (Table 4.3 and Table 4.4). Genes ranked in the top 100 for either library cover the statistically significant differences in transcript ratios between libraries.

4.3.8 Gene ontology analysis

Gene ontology (GO) classifications provide consistent nomenclature for the description of gene function (Harris *et al.*, 2004). GO functional classifications for the *S. nodorum* gene set (courtesy of James Hane, ACNFP, Murdoch University, Australia) were combined with the EST abundance data for each library. Some genes contained multiple GO classifications, in these cases all were used for the analysis.

A total of 7886 ESTs belonged to a unigene with a GO classification, leaving 4342 ESTs or 35% of the total, without an annotated GO function (Table 4.5 A). Among the unigenes themselves, 1299 (58%) contained an GO match, while 953 (42%) did not (Table 4.5 B). All GO annotations were grouped under the three main classifiers, biological processes, cellular components and molecular function (Table 4.5 C)

Table 4.3　1st

Table 4.3　2nd

Table 4.3 3rd

Table 4.3 4th

Table 4.4 1st

Table 4.4 2nd

Table 4.4 3rd

Table 4.4　4th

Table 4.5

Functional categories were compared across the two libraries to determine what groups were most upregulated *in planta* and *in vitro*. A P value was calculated, using the method of Audic and Claverie (1997), which described the probability that the EST abundances in both libraries were equal for a particular GO classification. GO classifications upregulated *in planta* and *in vitro* were ranked on the basis of the P value within the three main groups, biological processes (Table 4.6), cellular components (Table 4.7) and molecular function (Table 4.8), the most statistically significant changes were recorded.

Key biological processes upregulated in the *in planta* library were; protein biosynthesis and processing, xylan catabolism, cellulose catabolism and polysaccharide metabolism. Key biological processes upregulated in the *in vitro* library were; lipid metabolism, malate metabolism, the TCA and glyoxylate cycles, cell cycle and cell division.

Cellular components is the second major class of GO terms. Examples upregulated in the *in planta* library were; the ribosome and the small ribosome subunit. Other upregulated classes were not statistically valid. Key cellular components upregulated in the *in vitro* library were; the peroxisome, the glyoxysome, nucleus, cytosol and mitochondrion.

Key molecular functions (the third major class) upregulated in the *in planta* library were; hydrolysis of o-glycosyl compounds, and arabinofuranosidase activity, serine-type endopeptidase acitivity and peptyl-prolyl cis-trans isomerase activity.

Key molecular functions upregulated in the *in vitro* library were; lactate and malate dehydrogenase activities, isocitrate lyase activity and acyltransferase activity.

Table 4.6

Table 4.7

Table 4.8

4.3.9 Candidate selection

The top 100 upregulated genes *in planta* were examined to choose gene candidates for more accurate expression analysis. They were chosen on the basis of two criteria; (1) they were upregulated *in planta* and (2) mutants lacking the gene were predicted to have a detectable phenotypic difference when compared to SN15. Eight genes were selected for further study (Table 4.9).

The candidate genes were; SNOG_16499 (26 *in planta*; 0 *in vitro*), SNOG_03877 (24 *in planta*; 0 *in vitro*), SNOG_00557 (21 *in planta*; 0 *in vitro*), SNOG_12085 (11 *in planta*; 0 *in vitro*), SNOG_8496 (10 *in planta*; 0 *in vitro*), SNOG_12441 (10 *in planta*; 0 *in vitro*), SNOG_14274 (9 *in planta*; 0 *in vitro*) and SNOG_11873 (7 *in planta*; 0 *in vitro*). The candidate genes covered a range of EST abundances and cellular functions.

4.3.10 Gene expression of candidate genes during sporulation *in vitro* and *in planta*.

Transcript levels from the eight candidate genes were determined by a more accurate method, quantitative PCR (qPCR). Transcripts were quantified in cDNA pools made from RNA isolated from *in vitro* and *in planta* growth of SN15 at early and late infection time points. Three biological replicate RNA samples were pooled prior to cDNA synthesis, and two technical replicates were analysed for each treatment. This was due to monetary limitations. *In vitro* cultures sampled at 4 dpi did not contain any pycnidia, whereas cultures sampled at 18 dpi were heavily sporulating with many pycnidia. To isolate RNA from both early and late infections of wheat, an SN15 infection latent period assay was used to provide *in planta* infection transcripts. Lesions were excised after 8 dpi and 12 dpi, representing early and late infection stages.

Overall, the 4 tissue types represented clear states of non-sporulation and sporulation during both *in vitro* and *in planta* growth.

Table 4.9

Figure 4.3

In order to reveal which genes were upregulated during sporulation *in planta*, transcript levels were calculated by a qPCR amplification of cDNAs. The concentration of transcript was expressed relative to that of actin (SNOG_01139). The quantification of transcript levels by qPCR provided precise transcript information compared to the more qualitative EST data.

Genes that matched the desired profile of upregulation during sporulation were; SNOG_00557, SNOG_03897, SNOG_12085, SNOG_14274 and SNOG_16499 (Figure 4.3). The most dramatic changes between sporulation *in vitro* and *in planta* were seen in SNOG_00557 (350-fold change), SNOG_03897 (40-fold change) and SNOG_16499 (6.6-fold change).

SNOG_00557 transcripts *in vitro* at 4 dpi (0.0013/actin) were very low and almost unchanged at 18 dpi (0.0030/actin) (Figure 4.3 A). Interestingly, *in planta* transcription at 8 dpi was upregulated over 600 times that of 4 dpi *in vitro* (0.8173/actin) and at 12 dpi (1.0568/actin) was an almost 350 fold increase over *in vitro* 18 dpi. The overall profile was very low expression *in vitro* and high expression *in planta* with small changes within sporulating and non-sporulating situations for each growth environment.

SNOG_03877 transcript *in vitro* at 4 dpi (0.0049 /actin) was almost unchanged at 18 dpi (0.0031/actin), whereas *in planta* transcript at 8 dpi was 0.0269/actin and at 12 dpi almost 5 times higher at 0.1263/actin (Figure 4.3 B). The overall profile was low expression *in vitro* and high expression only during sporulation *in planta.*

SNOG_08496 transcript *in vitro* at 4 dpi (0.1754/actin) was up 1.5 fold at 18 dpi (0.2662/actin), whereas *in planta* transcript at 8 dpi was 0.1449/actin and almost unchanged at 12 dpi (0.1697/actin) (Figure 4.3 C). The profile *in vitro* was moderate expression with a small increase at 18 dpi. *In planta* samples showed little change between sporulating and non-sporulating situations. This gene was not correlated with sporulation *in planta*.

SNOG_11873 transcription *in vitro* at (4 dpi, 0.019/actin) was almost unchanged by 18 dpi (0.026/actin), whereas *in planta* transcription at 8 dpi was 0.014 /actin and at 12 dpi over 3-fold higher at 0.049 /actin (Figure 4.3 D). There was a small increase in SNOG_11873 transcription between the two *in vitro* samples, but during growth *in planta* there was a large increase in transcription during sporulation.

SNOG_12085 was observed to be upregulated during growth *in planta* compared to growth *in vitro* (Figure 4.3 E). Transcript *in vitro* at 4 dpi (0.108/actin) was almost unchanged at 18 dpi (0.177/actin), whereas *in planta* transcript at 8 dpi was 0.187/actin and at 12 dpi was 2-fold higher at 0.420 /actin. The overall profile was moderately low expression in both *in vitro* conditions; and an increase *in planta* during sporulation. There very little change in SNOG_12441 transcription between any of the growth states tested (Figure 4.3 F). Transcript *in vitro* at 4 dpi (0.107/actin) was almost unchanged at 18 dpi (0.132/actin), and *in planta* transcript at 8 dpi was 0.108 /actin and at 12 dpi was slightly up at 0.138/actin. The overall profile showed a reasonably stable low level of expression over all conditions.

There was generally higher expression of SNOG_14274 *in planta* compared to *in vitro* growth (Figure 4.3 G). Transcript *in vitro* at 4 dpi (0.053/actin) decreased by 18 dpi (0.029/actin), whereas *in planta* transcript at 8 dpi was 0.143/actin and at 12 dpi was even higher at 0.176/actin. There was a 6-fold increase in transcription between *in vitro* sporulation and *in planta* sporulation.

SNOG_16499 was expressed more during growth *in planta* compared to growth *in vitro* (Figure 4.3 H). There was low levels of transcript *in vitro* at 4 dpi (0.037/actin) and was less than 2-fold higher by 18 dpi (0.068/actin), whereas *in planta* transcript at 8 dpi was much greater at 0.346/actin and increased again at 12 dpi to 0.445/actin. If both "sporulation" samples are compared, expression was over 6-fold higher during sporulation *in planta* then *in vitro*. The overall profile was low expression *in vitro* and high expression *in planta* with smaller changes between the two sporulation situations. After consideration of the qPCR data, up to five genes were considered as possible candidates for gene knockout studies based on their expression profiles, however, only the gene SNOG_14274 was selected for further analysis by gene replacement mutagenesis as it was predicted to be the most likely to be involved in the process of sporulation.

4.3.11 Annotation of SNOG_14274

The automated gene annotation for SNOG_14274 was checked by using a BlastX search with the sequence from the locus to find matching translations in the Genbank non-redundant database. Secondly, the predicted amino acid sequence was used as a BlastP query against the Genbank non-redundant CDS database. The matches from the translated sequence matched the matches from the automated deduced amino acid sequence. This indicated the automatic annotation was suitable for use as the defined

gene sequence. SNOG_14274 contained one intron, which was confirmed by alignment of the predicted cDNA with matching EST sequences. BlastP comparison of SNOG_14274 to the non-redundant CDS database revealed many hits with a high degree of similarity. The first hit with an annotated function was a L-arabinitol 4-dehydrogenase isolated from *Hypocrea jecorina* (AAL08944) (Richard *et al.*, 2001). The alignment had a score of 522 bits, an expect score of 1e-147, and was 71% identical at the amino acid level. Of the top 10 hits, four proteins were arabinitol 4-dehydrogenases and the other six were hypothetical translations. The gene was considered most likely to encode an arabinitol 4-dehydrogenase and was named *Abd1*. A BlastX search of the *S. nodorum* genome revealed that *Abd1* was likely to be the only gene encoding an arabitol 4-dehydrogenase.

4.3.12 Deletion of the gene *Abd1*, encoding arabitol 4-dehydrogenase.

To determine whether *Abd1* is required for sporulation in *S. nodorum*, the gene was removed from the wild-type strain SN15 using targeted gene replacement. The deletion construct was created using the overlap PCR method, as described by Solomon *et al.* (2006d) (Figure 4.4).

A 937 bp region 5' of the *Abd1* start codon was amplified with AbdKO5'F and AbdKO5'R primers and a 820 bp region 3' of the *Abd1* stop codon was amplified using primers AbdKO3'F and AbdKO3'R. The two flanks were fused either side of a 2424 bp phleomycin resistance cassette using overlap PCR. The resulting amplicon was used to transform SN15 protoplasts.

Genomic DNA isolated from 19 phleomycin-resistant colonies was used to check the nature of the recombination event. The entire *Abd1* locus in each mutant was amplified.

Figure 4.4

by PCR using primers AbdScrnF and AbdScrnR. Two products were predicted, a 4495 bp amplicon indicated the *Abd1* locus had been replaced by the phleomycin cassette, while a 3028 bp amplicon indicated that the *Abd1* locus was intact. Selected strains representing suspected knockout and ectopic integration events (abd1-6, abd1-8, abd1-10, abd1-12, abd1-14, abd1-18 and abd1-19) were further analysed by Southern blot. (Figure 4.5) Genomic DNA (10 µg) was digested with *Bgl*II and hybridised with a Dig-labelled probe made from the Abd 3' flank region (PCR amplified with primers AbdKO3'F and AndKO3'R). Presence of a wild-type *Abd1* locus would result in a 4.5 kb restriction fragment hybridised with the probe, while a knock-out strain would result in a 5.9 kb restriction fragment hybridising. The Southern blot showed that all of the strains had undergone single recombination events at the *Abd1* locus, and all but one were derived from single integration events. *Abd1*-10 showed what appeared to be a tandem integration at the *Abd1* locus. No strains were identified with the knockout construct integrated at an unknown locus, and hence no 'ectopic' mutants could be used as controls.

4.3.13 Pathogenicity of *abd1* strains in a detached leaf assay

Sporulation and pathogenicity of *abd1* mutants was tested in a detached leaf assay, on wheat cv. Amery.

4.3.13.1 Pycnidia development

Pycnidia production was visibly reduced in all three *abd1* strains (Figure 4.6 A). Average numbers of pycnidia per leaf for the strains were as follows: SN15, 45; *abd1*-12, 32; *abd1*-14, 36; *abd1*-19, 33; mock, 0 (Figure 4.6 B). The largest reduction in pycnidia number was observed in strain *abd1*-12, which had a 28 % fewer pycnidia

compared to SN15. Statistical comparison of means by t-test showed the probability was low that pycnidia production in SN15 was equivalent to that in *abd1*-12 (P = 0.05),

Figure 4.5

to *abd1*-14 (P = 0.11) and to *abd1*-19 (P = 0.04). All knockouts had significantly reduced sporulation at a 90 % confidence and abd1-12 and abd1-19 were also considered reduced at 95 % confidence.

4.3.13.2 Lesion development

Average lesion sizes produced by the strains were as follows: SN15, 13.4 mm; *abd1*-12, 11.1 mm; *abd1*-14, 12.3 mm; *abd1*-19, 12.0 mm; mock, 0 mm (Figure 4.6 B). Lesion size was slightly reduced in *abd1* mutants, but the difference was only significant at 90% confidence for strain *abd1*-12. Lesion sizes followed similar trends to pycnidia numbers, where *abd1*-12 had the most reduced lesion size relative to SN15, 17% less. The host tissue infected with *abd*1 strains quickly turned a brownish-yellow colour in a similar manner to the SN15 infections.

Further characterisation of the *abd*1 mutants was not possible due to time constraints.

4.4 Discussion

A cDNA library was constructed from transcripts isolated from a late-stage *in planta* infection. Approximately 10,000 clones were sequenced in two directions, along with another library derived from an *in vitro* SN15 culture. The two libraries were used to determine what genes were upregulated during growth *in planta* versus growth *in vitro*. Eight genes abundant in the *in planta* library had their expression levels accurately determined using quantitative PCR, which confirmed that four of those were highly upregulated during growth *in planta*. An arabitol 4-dehydrogenase (*Abd1*) gene was mutagenised in SN15 by targeted replacement. Mutants lacking *Abd1* had slightly reduced sporulation *in planta*, two of the three were significantly different from wild-type at a 0.05 confidence limit.

Figure 4.6

4.4.1 Analysis of the two cDNA libraries.

Over 20,000 raw sequences were created from both libraries, resulting in over 12,000 ESTs that could be aligned with the *S. nodorum* predicted protein set. Both libraries were good representations of transcription at the time as redundancy was low, with only 427 of 2252 genes represented in both libraries. The sequence was of good quality, with both libraries containing a median sequence length of over 500 bp after trimming. Both libraries appeared to contain bimodal distributions of sequence length. This was most probably caused by the sequencing method. Each clone was sequenced in forward and reverse directions, and primer positions were at different distances from the cloned insert in each direction.

The distribution of unigenes showed that both libraries were highly distinct, only 427 of the 2252 unigenes were present in both libraries. Conversely, if the number of ESTs per unigenes were counted, those 427 genes produced almost half of the total ESTs. This observation may be because highly expressed genes were more likely to be found in two libraries, in this theory, if twice as many ESTs were sequenced the number of unigenes found in both libraries would increase. A similar division between *in vitro* grown libraries and *in planta* libraries was reported in *Mycospharella graminicola* and a study of seven libraries produced from *Fusarium verticillioides* found that from a total of 11,119 unigenes, 6048 were found in only one library, and 1130 were represented in four or more libraries (Brown *et al.*, 2005a; Keon *et al.*, 2005). These studies confirm the need for multiple libraries representing multiple growth states when using ESTs to characterise a fungal genome.

4.4.2 Analysis of genes up-regulated in each library

Genes present in each library were analysed in two ways, firstly, genes were ranked on the basis of EST abundance in each library, and secondly, on the basis of GO classifications within those ESTs. The statistical probability that any observed differences were due to random chance was used to rank the gene lists. Both bioinformatic analyses revealed similar characteristics within each library.

4.4.2.1 GO annotation of EST libraries.

GO classifications were assigned to each cluster of ESTs to provide a broader view of the biological differences revealed by the libraries. Over half of the ESTs did not have a significant match to a GO classification. This level of unidentifiable transcripts is quite common in EST libraries from fungi. A comparative study involving ESTs from 11 different fungi showed over 50 % of the ESTs from each fungus could not be assigned a functional category (Soanes and Talbot, 2006). Also, approximately 35-45% of the ESTs characterised in a study of the wheat pathogen, *M. graminicola* remained unidentified (Keon *et al.*, 2005).

4.4.2.2 Genes up regulated *in planta.*

The *in planta* library was derived from lesions isolated from infected wheat plants, with plant tissue that was almost entirely dead and fungal colonies that were in both early and late stages of infection and sporulation. The main gene types upregulated *in planta* were ribosome associated proteins, proteolytic, and plant cell-wall degrading enzymes.

ESTs from SNOG_01849, which is similar to chymotrypsin, were found 72 times in the *in planta* library, and not at all in the *in vitro* library. In addition, a gene encoding a MFS peptide transporter (SNOG_09813) was upregulated in the *in planta* library (7:0).

These results may relate to the use of degraded plant proteins as an energy source *in planta*. A search of the predicted protein set for *S. nodorum* SN15 showed that the upregulated gene (SNOG_09813) was not *Ptr2*, a transporter previously shown to be required for the utilisation of di/tripeptides (Solomon *et al.*, 2003). There are at least eight SN15 genes related to *Ptr2*, of which only one (SNOG_09813) was found in the upregulated *in planta* gene set. These genes showed up in the GO annotations, with classifiers 'proteolysis' and 'oligopeptide transport' and were identified as upregulated in the biological processes field, along with the 'endopeptidase' group in the molecular functions category.

Twenty five of the top 100 genes upregulated *in planta* library were linked to the ribosome and protein synthesis, including SNOG_03556, SNOG_00514, and SNOG_04727, which together had 137 ESTs in the *in planta* library, and 1 in the *in vitro* library. These genes encode ribosomal protein subunits rather than structural RNAs. They indicate that protein biosynthesis is very active during the infection, perhaps indicative of high protein turnover in a highly stressful environment. The GO annotations identified 'protein synthesis' and 'protein folding' within biological processes, and four different ribosome-related groups in the cellular components category. Another reason for the increased protein synthesis during growth *in planta* could be that the secretion of proteins into the extracellular environment is stimulated during *in planta* growth. The observed upregulation of transcripts involved in protein synthesis is similar to a report about three cDNA libraries from *Mycosphaerella graminicola* (Keon *et al.*, 2005). They observed that a library constructed from a late-stage infection of wheat, contained an increased representation of genes predicted to be involved in protein biosynthesis, relative to libraries from budding growth and

pseudohyphal growth. The late-stage infection sampled was undergoing asexual sporulation, it was suggested that protein synthesis and protein fate functions may have been linked to the high rate of pycnidia biogenesis.

Wheat cell wall degradation is a key part of the infection process, several genes related to degradation of carbohydrate-polymers were upregulated in the *in planta* library. GO terms' xylan degradation', 'cellulose degradation', 'polysaccharide catabolism', 'arabinofuranosidase' and 'o-glycosyl hydrolysis' were increased in abundance relative to the *in vitro* library. These classifiers accounted for genes such as SNOG_00557 an Arabinofuranosidase, and SNOG_07191 a potential cell wall glycosyl hydrolase, being solely found in the *in planta* library.

4.4.3 Genes up regulated *in vitro*.

The *in vitro* library was characterised by genes involved in peroxisomal function and lipid breakdown. This was expected as the culture the library was derived from was grown on oleate as the carbon source. Several genes related to peroxisomal function were identified, including; SNOG_14143, similar to Pox18 and SNOG_06131, related to a 3-ketoacyl-coa thiolase (Erdmann and Kunau, 1994; Szabo *et al.*, 1989). These genes are most likely responding to the use of oleate as the sole carbon source, which would require lipid catabolism in the peroxisome. Lipid degradation is usually accompanied by upregulation of the glyoxylate pathway, to permit growth on the acetyl-CoA produced by beta-oxidation of fatty acids (Stryer, 1996). Genes similar to isocitrate lyase (34:0) and malate dehydrogenase (66:0) were also highly abundant in the *in vitro* library and absent from the *in planta* library. GO classifications provided a similar pattern, the peroxisome and the glyoxysome were identified as cellular

components upregulated in the *in vitro* library, while malate and lipid metabolism were identified as upregulated biological processes in the GO classifications.

In summary, the *in planta* library was dominated by expression of genes relating to degradation of host proteins and cell wall carbohydrates, while also contained increased abundance of genes associated with protein synthesis. The *in vitro* library showed gene expression changes characteristic of lipid degradation, consistent with the utilisation of oleate as the carbon source.

4.4.4 Quantitative expression analysis of genes upregulated *in planta*.

Eight candidate genes were further characterised by quantitative PCR, to confirm the expression levels *in planta* and *in vitro*. This time, however, expression was determined from sources clearly delineating sporulating and non-sporulating growth, both *in planta* and on defined media. The transcript abundances observed with qPCR were mostly in agreement with the qualitative data from the EST libraries, indicating the two libraries are genuinely representative of the transcript levels in the cell. SNOG_01139 (actin) was used to normalise expression levels in the qPCR experiments. The ESTs mapped to this gene were present in approximately equal amounts in the *in planta* (8 ESTs) and *in vitro* (10 ESTs) libraries. This validated the use of this gene to normalise the quantitative PCR abundances.

The genes with the lowest P values based on EST abundance showed the greatest upregulation *in planta* when analysed by qPCR. Those genes shown to only be marginally or not at all regulated, had EST P values larger than 0.0001. While this is a low probability, when the large sample size of over 2,200 genes is considered it is not as significant as would be the case in a smaller experiment. For example, a P value of 0.01

in a sample that large would be calculated to result in 22 false-positives. The most upregulated genes were SNOG_00557, SNOG_03877, SNOG_14274 and SNOG16499.

SNOG_00557 was predicted to encode an arabinofuranosidase, a cell wall degrading enzyme. It was the most highly expressed gene in the qPCR study, with an abundance equal to that of actin, during sporulation *in planta*. The upregulation during infection was huge, up to 600-fold that observed during *in vitro* growth. This matches the hypothesised role of the enzyme in breaking down the plant cell wall, providing a significant energy source once host cell death began. Interestingly, both SNOG_12085 (putative UDP-glucose 4-epimerase) and SNOG_14274 (putative arabitol 4-dehydrogenase) were upregulated during sporulation *in planta*, compared to *in vitro* sporulation. These three upregulated genes appear to have connected roles in the metabolism of arabinan, or xylan, carbohydrate derivatives. Arabinofuranosidase enzymes can produce arabinose from arabinan or xylose from xylan. Both arabinose and xylose are within two enzymatic steps from arabitol, produced by arabitol 4-dehydrogenase. These three upregulated genes may highlight a pathway used to utilise the abundant plant cell wall carbohydrates.

4.4.5 Sporulation of *abd1* mutants.

SNOG_14274, renamed *Abd1,* was found to share significant sequence similarity with arabitol 4-dehydrogenase genes. It was deleted from the SN15 genome by targeted gene replacement. *Abd1* was selected from the four upregulated genes because it was most likely to provide a distinguishable sporulation phenotype. The other three genes were thought to encode enzymes that either wouldn't directly contribute to the developing spore, or did not have a clear function at all. Mannitol, a six carbon polyol, has previously been shown to be required for asexual sporulation of *S. nodorum* (Solomon

et al., 2006e). Arabitol, a four carbon polyol, has been identified in *S. nodorum* and other fungi (Jennings, 1984; Link *et al.*, 2005; Pail *et al.*, 2004; Solomon *et al.*, 2005a).

The gene *Lad1*, in *Hypocrea jecorina* was shown to be required for growth on L-arabinose in culture, and for full growth on L-arabitol (Pail *et al.*, 2004). Some redundancy with the *Xdh1* gene product was described, as when both *Lad1* and *Xdh1* were mutated growth on arabitol was completely halted.

In *Uromyces fabae*, a biotrophic pathogen of broad bean, an arabitol dehydrogenase gene *Ard1* was characterised (Link *et al.*, 2005). Transcripts were detected in the uredospore with highest expression present in the haustorium feeding structure, enzyme activity was only detected during *in planta* infection. Immuno-fluorescence microscopy revealed the Ard1p enzyme was localised to the haustorium during infection.

These results suggested that in *S. nodorum Abd1* may be an involved in the metabolism of degraded plant cell wall components, such as arabinan and xylan. It represented an ideal target for mutagenesis in *S. nodorum*.

Loss of *Abd1* was found to slightly reduce pycnidia formation during a DLA. Three independent *abd1* mutants all showed a reduction in pycnidia numbers at 7 dpi. As the reduction was slight, it suggests that arabitol may act in an additive manner with other compatible osmolytes in *S. nodorum*. The osmolytes mannitol, arabitol, and trehalose have been identified in *S. nodorum*, with arabitol present at lower concentrations than mannitol and trehalose (Solomon *et al.*, 2005a). It appears that while arabitol biosynthesis may have role in sporulation, it is as a minor requirement. It may be that

arabitol biosynthesis is required during early growth *in planta*, and in *abd1* strains the leaf is not colonised as densely as in wild-type infections, leading to a delay in sporulation initiation. In addition, any functional redundancy amongst the *S. nodorum* polyol dehydrogenases could reduce the impact of an *Abd1* deletion. Interestingly, although there was an observed drop in sporulation rates *in planta*, spore production *in vitro* was not seen to be effected.

Further work is required to elucidate the reason for a reduction in sporulation during a DLA. Firstly, an *abd1* mutant should be genetically complemented by reintroduction of the *Abd1* gene to confirm the sporulation phenotype can be rescued. Secondly, measurement of arabitol levels in the *abd1* mutants during infection and growth *in vitro* would help pinpoint when the lack of arabitol, if any, occurred. Arabitol has been shown to complement the loss of glycerol in an *A. nidulas gldB* mutant during osmotic challenge (de Vries *et al.*, 2003). Also, in *Hypocrea jecorina*, arabitol 4-dehydrogenase was important for growth on a variety of hexoses (Pail *et al.*, 2004). Perhaps arabitol has roles in osmo-tolerance and energy uptake from the host, which help to boost growth and sporulation *in planta*. Future work should try and determine if there is a functional synergy between fungal polyols during growth *in planta*, where the population of different polyol species changes as the pathogen adapts to different stages of infection.

Chapter 5

Metabolomics of *in planta* sporulation.

5.1 Introduction.

5.1.1 Metabolomics.

Physiological development phenomena, such as sporulation, have historically been investigated from a gene and mRNA-based standpoint. Unfortunately, that approach will not explain phenomena that depend on regulation at the translation or enzyme activity level. For example, mRNA abundance has been shown in many cases to be unreliable for the prediction of protein abundances (Griffin *et al.*, 2002; Gygi *et al.*, 1999). A cross-platform approach is more likely to capture all of the biological changes that give rise to a particular developmental shift.

The field of metabolomics is ideally suited to the exploration of fungal development as it can capture and quantify 100's of metabolites simultaneously. The term metabolome refers to the entire metabolite complement of a biological system at a particular time (Dunn and Ellis, 2005). Thus, metabolomics is the study of the metabolome. The technologies used for metabolomics are diverse and most have been established for a considerable time. The application of such techniques to rapidly describe the metabolic phenotype of a biological systems in such detail is a recent development (Fiehn, 2002). Metabolomics is particularly useful to distinguish biological samples by their metabolite phenotype. For example, a study of plant development using both transcript and metabolite profiling showed that metabolite levels were more successful at differentiating the tissue types analysed (Urbanczyk-Wochniak *et al.*, 2003).

The metabolite complement found within a living organism is hugely diverse. Even in *S. cerevisiae*, a single-celled eukaryote, as many as 600 metabolites have been identified

thus far (Forster *et al.*, 2003). The metabolome of plants is far larger and includes a vast array of secondary metabolites. The plant metabolome has been estimated to involve up to 200,000 different metabolites (Fiehn, 2002). The first attempt to analyse the total metabolite complement of a biological sample was probably Pauling (1971), who used gas chromatography flame ionisation detection to detect 280 different volatile substances in human urine. Pauling did not use the term metabolome, it was first used in literature by Oliver *et al.* (1998) in a paper discussing how the transcriptome, proteome and metabolome could be integrated with the genome sequence of *S. cerevisiae* to gain insight to how the eukaryotic cell works.

5.1.1.1 Metabolomics and metabonomics.

Metabonomics is a term used mostly in medical research to describe a more defined field of metabolomics. It usually describes the analysis of whole organisms including tissue specific and time-related data, often involving analysis of urine and blood plasma (Nicholson *et al.*, 1999). It also usually relates to the monitoring of pharmaceutical-induced biochemical changes. Outside of the medical field, the term 'metabolomics' is more commonly used to describe the same type of tissue-specific and temporal studies (Roessner *et al.*, 2006). A search of the Pubmed literature database with the keyword "metabolomics" retrieved 372 articles, while "metabonomics" retrieved 160 articles. The current situation is one where both terms are common, but the definitions are overlapping and are sometimes used interchangeably.

5.1.2 Technologies.

The technology for metabolomics usually involves either of two main groups, nuclear magnetic resonance (NMR) spectroscopy, or gas/liquid chromatography mass spectrometry (GC-MS and LC-MS) techniques. Less common technologies are

capillary electrophoresis-mass spectrometry (CE-MS), Fourier transform infrared (FT-IR) spectroscopy and Fourier transform ion cyclotron resonance mass spectroscopy (FT-ICR or FT-MS). These technologies have generally found a niche in the field of metabolomics, and are described below.

5.1.2.1 Nuclear magnetic resonance spectroscopy.

The technique of nuclear magnetic resonance (NMR) spectroscopy was developed in 1947, independently by Felix Bloch and Edward Mills Purcell (Bloch, 1946; Purcell *et al.*, 1946). Its use for metabolite quantification was pioneered by Nicholson *et al.* who reported the concentrations of amino acids, fatty acids, glucose, lactate, creatine, choline, citrate and D-3-hydroxybutyrate from human blood (Nicholson *et al.*, 1983). The main advantages of NMR metabolomics are the minimal sample preparation required, non-destructive sampling of metabolites without derivatisation and the potentially high through-put of samples (200-300 per day). The sensitivity of NMR is dependant on the analysis time and the equipment used, but is generally lower than quadrupole or time-of-flight mass-spectrometers (Keun *et al.*, 2002).

Most examples of NMR metabolomics studies involve 'fingerprinting' samples without in depth analysis of individual metabolites. NMR 'fingerprints' can be used to quickly identify disease states or genotypes. For example: it was used as a non-invasive screen for drug toxicity (vasculitis) in rats after treatment with a pharmaceutical (Robertson *et al.*, 2001). NMR of *Aspergillus nidulans* was used as a general method to determine the specificity of lead-chemicals in a drug discovery program and also to investigate general carbohydrate metabolism (Dijkema *et al.*, 1985; Forgue *et al.*, 2006). Also, it has been used to identify wild-type and transgenic *Nicotiana tabacum* plants (Choi *et al.*, 2004a).

5.1.2.2 Gas chromatography-mass spectrometry.

Gas chromatography-mass spectrometry (GC-MS) is the most common technology used for metabolomics. A gas chromatograph is used to separate the metabolites, followed by mass spectrometry to detect and identify the composition of the resolved metabolite. In this technique derivatised metabolites are vaporised in the sample inlet and carried into the GC column. Columns usually have polar stationary phase, to best separate polar metabolites and use an inert gas, such as helium, as the mobile phase. Resolved metabolites can be ionised by a number of methods including, chemical ionisation, flame ionisation, and electron impact ionisation. Mass spectrometry is the most common method of detection in metabolomics, as it gives a high level of metabolite identification and deconvolution. Mass spectrometers usually incorporate quadrupole or time-of-flight (TOF) ion analysers. TOF analysers offer much faster scan speeds than quadrupole analysers, typically around 100 scans per second. This speed advantage means chromatography runs can be shorter (roughly one quarter the time) and peaks will still be captured as distinct entities by the TOF analyser.

The reproducibility of GC-MS methods is the most useful attribute of the system. It allows retention times of metabolites to be replicated on different systems, and wide-scale sharing of retention time and mass spectral data on metabolites (Schauer *et al.*, 2005). Many commercial and public domain metabolite databases exist to aid metabolite identification with GC-MS. These include the NIST mass spectral library of over 20,000 metabolites and several libraries available from the Golm Metabolome consortium, totalling around 2000 different entries (Kopka *et al.*, 2005). The use of these databases means each lab does not have to analyse its own suite of authentic standards, saving thousands of dollars and countless hours of analysis.

The main limitation of GC-MS is the requirement to derivatise metabolites prior to analysis, and the inability to detect large, or non-volatile metabolites. It is best suited to the analysis of small-medium polar primary metabolites, such as amino acids, sugars, carboxylic acids, and fatty acids.

Derivatisation is performed to volatilise and stabilise metabolites for separation by gas chromatography. It typically involves the addition of trimethylsilyl groups to acid labile protons of the metabolite. As well as trimethylsilylation, keto or oxo groups on metabolites are usually oximated to prevent enolisation reactions and to reduce the complexity of the chromatogram (Halket *et al.*, 2005). Typically gas chromatography with a quadrupole mass detector will resolve 100-200 peaks from a plant sample. The use of a time-of-flight mass spectrometer along with low-bleed columns can increase this to over 300 peaks. The current leading-edge technology for GC-MS is the GCxGC MS system that couples two GC columns in series. The first column is a typical polar GC column, while the second column is usually a much shorter non-polar column. The extra selectivity results in far greater resolution of peaks and allows resolution of compounds that would normally co-elute. In a study of spleen tissue from obese mice, 1200 compounds were resolved by GCxGC-TOF, compared to 500 resolved by one-dimensional GC-TOF (Welthagen *et al.*, 2005).

Roessner *et al.* (2000), described in detail a new method for GC-MS analysis of potato tubers. The method has since become a widely used basis for analysis with GC-MS quadrupole systems in the field of plant metabolomics. Over 150 peaks were integrated, including 77 identifiable metabolites. The reported standard deviation for the entire

procedure was 6% or less for most compounds tested. Since then, studies have been published on *Arabidopsis thaliana* (Fiehn *et al.*, 2000), *Lotus japonicus* in symbiosis with *Mezorhizobium loti* (Colebatch *et al.*, 2004; Desbrosses *et al.*, 2005) and apricot fruit (Katona *et al.*, 1999).

5.1.2.3 Liquid chromatography-mass spectrometry.

Liquid-chromatography mass spectrometry (LC-MS) is most commonly used for the analysis of large metabolites or non-volatile species. The sample does not require derivatisation prior to analysis, simplifying procedures. Detection is most commonly provided by electrospray ionisation-mass spectroscopy. Software for comparison of chromatograms, deconvolution of overlapping peaks, and for comparison of mass spectra are not as advanced as those available for GC-MS. LC-MS is the technique of choice for the analysis of complex plant secondary metabolites.

5.1.2.4 Minor platforms for metabolomics.

Minor platforms include capillary electrophoresis-mass spectroscopy (CE-MS), Fourier transform-infrared spectroscopy (FT-IR) and Fourier transform-ion cyclotron mass spectroscopy (FT-ICR). The main advantage of CE-MS is the ability to separate almost any charged metabolite with very high resolution, without prior derivatisation. It has been used to identify primary metabolites in rice (Sato *et al.*, 2004) and over 1600 compounds in *Bacillus subtillus* (Soga *et al.*, 2003). It is not however, commonly cited in the literature in relation to metabolomics.

FT-IR is used to determine a single mass spectra for a particular sample. Individual metabolites in a sample are not usually resolved prior to the spectra being collected. FT-IR has been used to rapidly distinguish fungal species without metabolite-specific

analysis (Fischer *et al.*, 2006). It tends to be used less than NMR for use as a metabolic fingerprinting technique, but can offer extremely high throughput of samples (1000s per day) (Dunn and Ellis, 2005). The main limitation related to its use for metabolomics research is the difficulty in resolving and identifying individual metabolites.

Fourier transform ion cyclotron resonance mass spectroscopy (FT-ICR) is a very high resolution method for determining the mass-to-charge ratio of ions. FT-ICR mass spectroscopy is an emerging technique in the field of metabolomics. The high-cost of instrumentation has been the main limitation of FT-ICR, but aside from that it offers great benefits. FT-ICR is a non-destructive method of determining ion masses and can be coupled to many established separation technologies such as liquid or gas chromatography. The main benefits of FT-ICR are that has the highest resolution (at least 0.0001 of a mass unit), lowest detection limits and greatest flexibility of all the mass-spectrometers (Brown *et al.*, 2005b). The extreme resolution of mass accuracy means that prior separation of complex mixtures is not required. Publications relating to FT-ICR metabolomics are rare, but it has been used to identify metabolites in strawberry fruit associated with its development (Aharoni *et al.*, 2002), and determine the metabolic changes in *A. thaliana* plants over expressing a MYB transcription factor (Tohge *et al.*, 2005). Over 1800 different metabolites were resolved in the *Arabidopsis* study, over six times the amount usually resolved by GC-MS. It is a very good technique for the identification of unidentified metabolites, because of its extreme mass accuracy. The use of FT-ICR will most likely increase steadily in the future as more instruments become available to researchers.

5.1.3 Metabolomics studies of fungi.

The first dedicated metabolomics study of a fungus was performed by (Villas-Boas *et al.*, 2005). They developed a novel GC-MS method for the detection of primary metabolites in yeast. They claimed metabolite measurements revealed a similar level of detail in comparison to gene expression analysis. They used a novel derivatisation technique, analysing methyl chloroformate derivatives. This is not a common technique, and limited the number of reference spectra available for comparison. The conclusions were mostly related to validation of various growth conditions and normalisation procedures. While the technique was novel for yeast metabolomics, it was largely redundant considering the more widely adopted plant GC-MS methods published earlier (Roessner *et al.*, 2000). In 2003, Nielsen and Smedsgaard published a study describing liquid chromatography methodology for the identification of 474 fungal metabolites from a variety of organisms {Nielsen, 2003 #604}. This has provided an excellent resource for the identification of key metabolites, focussing on those with toxic or bioactive properties.

Recently, FT-IR and ESI-MS based metabolomics of the plant host *Brachypodium distachyon* and fungal pathogen *Magnaporthe grisea* interaction showed a link between the phosphatidic acid phospholipid content of leaves and the degree of resistance to the pathogen (Allwood *et al.*, 2006). *Medicago truncatula* cell culture suspensions have been analysed by GC-MS and LC-MS to determine the metabolomic changes during abiotic and biotic stress (Broeckling *et al.*, 2005). Currently, the literature on experimental metabolomics is increasing slowly (Figure 5.1) but is likely to expand greatly over the next few years, particularly in the field of plant fungal interactions with projects initiated studying the plant-pathogens *Fusarium graminearum* and *Magnaporthe grisea*.

Figure 5.1

The metabolome of a plant-rhizosphere interaction was investigated using a model system of *Arabidopsis thaliana* and *Pseudomonas* spp. Metabolomic analyses were performed using HPLC and ESI-MS, revealing that flavonoids and lignins were the major metabolites released by colonised roots (Narasimhan *et al.*, 2003).

Fusarium head blight resistance has been investigated at the metabolome level by GC-MS analysis of Fusarium-infected spikelets from resistant and susceptible cultivars (Hamzehzarghani *et al.*, 2005). The study focussed on metabolites that were related to plant defence, rather than those that were produced by the pathogen. The infection was allowed to progress for 24 hours, only focusing on the initial response to infection. Although not a pathogen-plant interaction, the symbiotic relationship between *Lotus japonicus* and *Mesorhizobium loti* was investigated using GC-MS. It was found that among others, asparagine, mannitol, putrescine, octadecanoic acid and glycerol-3-phosphate were abundant in the nitrogen-fixing nodules.

5.1.4 This study

There has not been any published attempt to analyse the metabolome of *S. nodorum* under any growth conditions. While wheat resistance to a fungal pathogen has been studied at a metabolome level, samples were collected at 24 hours, well before sporulation had occurred (Hamzehzarghani *et al.*, 2005). Here, the metabolome of *S. nodorum* and the wheat *Triticum aestivum cv.* Amery was investigated during the asexual life-cycle to reveal the key metabolic changes that occur before and during sporulation.

The platform chosen for metabolome analysis of *S. nodorum* sporulation *in planta* was gas chromatography mass spectrometry or GC-MS. This system allowed reproducible

separation of a wide range metabolites and provided a dual-sided system for identification of metabolites, using retention-time matching and mass-spectral-tag (MST) matching.

5.2 Materials and methods

5.2.1 Infected wheat leaves for GC-MS analysis

2-week old wheat cv. Amery plants were inoculated, incubated and excised as described for a latent period assay. Both SN15-infected and mock-infected plants were prepared. Five leaves were prepared per sample, and three biological replicates were prepared per time point. The entire leaf sample was collected at 8 dpi, 10 dpi and 12 dpi, at midday. Harvested leaves were snap-frozen in liquid nitrogen. Samples were stored at -70ºC until metabolites were extracted.

5.2.2 Extraction of metabolites for GC-MS analysis.

Harvested tissue was (10 to 100 mg fresh weight) disrupted in a mortar and pestle under liquid nitrogen. Ground tissue was added to a pre-weighed tube containing 1 mL methanol and 50 µL ribitol internal standard (1.3 mM), reweighed, vortexed briefly and then extracted at 70ºC for 15 minutes with shaking. Samples were subsequently centrifuged at 20,000 *g* for 3 minutes. The methanol phase was reserved and the pellet re-extracted with 500 µL H_2O and 375 µL chloroform for 5 minutes at 37ºC (with shaking) and then centrifuged at 20,000 *g* for 3 minutes. The polar phase was recovered and added to the reserved methanol extract. The remaining organic phase was discarded. The total polar extract was then lyophilised in a Maxi Dry Lyo (Heto Holten, Allerød, Denmark), prior to derivatisation.

5.2.3 Derivatisation of metabolites for GC-MS analysis

Oximation of carbonyl groups was performed by addition of 50 µL Methoxyamine-HCl (Sigma) (20 mg/ml in pyridine (Sigma)) to dried metabolites followed by incubation at 30ºC for 90 minutes with shaking. Trimethylsilyl (TMS) esters were then created by

addition of 80 µL *N*-Trimethylsilyl-*N*-methyl trifluoroacetamide (MSTFA) Sigma, and incubation at 37ºC for 30 minutes, with shaking. Samples were stored for 2 hours at room temperature before being analysed. Samples were sealed in glass GC-MS vials with a 100 µL insert and sealed with an Teflon crimp seal (Agilent, Palo Alto, CA, USA).

5.2.4 GC-MS analysis

The GC-MS equipment consisted of an Agilent 7680 autosampler, an Agilent 6890 gas chromatograph and an Agilent 5973N quadrupole mass spectrometer (Agilent, Palo Alto, CA, USA). The mass spectrometer was auto-tuned using perflurotrisbutylamine (PFTBA) according to the manufacturer's directions. A 30 m HP-50+ column with a 250 µm internal diameter and 0.25 µm film thickness was used for all gas chromatography (J&W scientific, Folsom, CA, USA). The column contains 50%-Phenyl)-methylpolysiloxane as the stationary phase. Derivatised metabolites were injected as 1 µL of sample, using a 1:20 split ratio.

5.2.4.1 Gas chromatography temperature settings

The injection temperature was 230ºC, interface temperature was 300ºC and the ion-source was held at 230ºC. Helium was used as the carrier gas, retention-time locking was not used. The GC oven temperature program was as follows: initial temp of 70ºC for 5 minutes, then increasing at 5 ºC/minute to 300 ºC held for 3 minutes. Data was collected after a 5 minute solvent delay. The total run time was 57 minutes. The methodology used was initially based upon the published method of Roessner *et al.* (2000).

5.2.4.2 Chromatogram deconvolution.

Routinely, 100 to 200 peaks were resolved per sample. Mass spectra and chromatograms were viewed or analysed using Chemstation software (Agilent, Palo Alto, CA, USA) and AMDIS software (Stein, 1999). Peak deconvolution settings on AMDIS included: adjacent peak substraction = 2, resolution = low, sensitivity = very low, shape requirements = medium, component width = 12. The software allowed overlapping peaks to be resolved, background-subtracted and deconvoluted, then mass spectral tags (MSTs) could be extracted for comparison to the MST database.

5.2.5 Metabolite identification.

The retention time of the peak was used to find co-eluting authentic standards, and then the mass spectral ion fragmentation pattern was also compared to match a standard to confirm the identity of a peak. Peak identification and area integration was performed using AMDIS software by comparison of peaks with GC-MS retention-time and mass-spectral databases. The Reverse spectra comparison method of the AMDIS software was used to judge the closeness of the MST match, along with manual inspection. A reverse quality score of 100 indicated a perfect match. The reverse algorithm only compares m/z abundances when the ion is present in both the unknown and the standard. This method ensures unrelated background ions will not always affect the accuracy of the comparison. Peak identities were manually screened for unreliable identifications. If a peak was only identified in one of the three biological replicates it was considered unreliable and excluded from further analysis.

Retention times peaks across replicates were averaged to provide a single representative elution time for all instances of that particular peak, and to allow automatic processing

of peak areas by statistical software. The best mass spectral match was recorded for each peak, along with the quality of the match.

5.2.5.1 GC-MS retention-time and mass-spectral libraries.

Three mass spectral libraries were used. The commercial NIST/EPA/NIH Mass Spectral Library (NIST, Gaithersburg, MD, USA), the Golm Metabolome Database annotated quadrupole GC-MS spectra library (Kopka *et al.*, 2005) and the ACNFP GC-MS TMS library.

NIST Mass Spectral Library.

This commercial NIST/EPA/NIH Mass Spectral Library was initially used for mass spectral matching of unknown compounds with Chemstation software. It was supplanted by the GMD annotated quadrupole GC-MS library and AMDIS software.

The GMD "Q_MSRI_ID" GC-MS library

The GMD "Q_MSRI_ID" GC-MS library was produced by the Golm Metabolome Databases Consortium, Golm, Germany. The library consists of annotated quadrupole GC-MS mass spectra and retention indices. It was used solely for mass spectral matching, as retention time indices were not comparable with RT values in this study. The following description of the contents was taken from the website www.csbdb.mpimp-golm.mpg.de where the library is available for download; *"Collection of 1166 identified or annotated mass spectral tags (MSTs). This collection contains 574 non-redundant MSTs of which 306 are identified. Mass spectra were manually generated from quadrupole GC-MS chromatograms of standard addition experiments or of biological samples. Each MST has an index number (MPIMP-ID) and a substance name. Non-identified MSTs are characterized by mass spectral match and name of best matching substance in square brackets".*

The ACNFP TMS library.

The ACNFP TMS library was produced by the ACNFP, Murdoch University, WA, Australia. The library consists of retention-time and MST information for authentic standards. The library described 50 authentic standards, producing 66 different peaks. The method to collect the library data was RT-locked and eluted mannitol at 24.30 minutes. The library is listed in the appendix.

The MPIMP "Pol_fa TMS" library.

The Pol_fa TMS Library was produced by the Max Planck institute of molecular plant physiology (MPIMP), Golm, Germany. It is available for download from the website www.mpimp-golm.mpg.de in the section "metabolite mass spectra library". The library describes 302 TMS-metabolites analysed by GC-MS with their retention times. These retention-times were generated using a method that was comparable with the methods used in this study. The method to collect the data was RT-locked and eluted mannitol at 24.2990 minutes. The library is listed in the appendix.

5.2.5.2 Retention time comparison between experimental and the "Pol_fa TMS" library.

Non-RT locked retention times such as those described in this chapter can be compared to the retention times from the ACNFP TMS library and the Pol-fa TMS library after transformation of the retention times. The formula

$Y = (1.0147*X) + 1.7033$ min was used to transform RT-locked values (X) to predicted non-RT-locked values (Y). Both observed RT-locked retention times and predicted non-RT locked retention times are listed in each library. Predicted values for non-RT locked experiments are not as reliable as using untransformed RT-locked data.

5.2.6 Metabolite abundance calculation

Peaks were integrated and areas calculated using AMDIS software. The metabolite area was divided by the area of the ribitol internal standard and by the tissue weight to give the normalized peak area. In the case of no recorded tissue weight or internal standard area, the peak area was expressed as a percentage of the total peak area for that chromatogram. If a metabolite was known to produce two peaks on the chromatogram, both areas were summed to give a total area for that metabolite, sometimes both the sum value and the individual peak values were analysed to provide as much information as possible.

5.2.6.1 Data processing

Data was processed using Excel software, (Microsoft) and JMP in 5.1 software, (SAS).

5.2.7 Hierarchical cluster analysis.

Hierarchical cluster analysis (HCA) is a method used to identify and group similar patterns within datasets. HCA begins by placing each variable into its own cluster and then in a stepwise manner, it combines the two most similar clusters, this continues until all clusters have been combined. The clustering process is drawn as a dendrogram. Clustering of biological samples was performed on normalised abundances for each metabolite in each sample. Clustering of metabolites was performed on the ratio of average normalised abundances of SN15-infected/mock-infected samples. The normalised abundances were averaged before the ratio was calculated. Missing values were replaced with a minimum value that corresponded to slightly less than the detection threshold. The ratios were log10 transformed to allow visualisation of the differences. Hierarchical cluster analysis was performed using JMP in 5.1 software, SAS. The clustering algorithm was Ward's minimum variance method of hierarchical clustering (Ward, 1963). The final number of clusters used was chosen from the dendrogram manually.

5.2.8 Principal components analysis.

PCA is a data reduction technique used to aid the analysis of complex datasets (Pearson, 1901). It is particularly appropriate for the analysis of metabolomics datasets because it can quickly determine which metabolites contribute most to the overall variation. The process assigns components to capture the variation and these components can be shown to define certain behaviours, such as specific organs, age of tissue or in this case, sporulation and infection progress. PCA was used to find which metabolites behaved similarly and which metabolites accounted for most of the changes in the profile. For example, a dataset containing abundances for 100 metabolites has 100 variables, but after application of PCA all of the variation could be described using only five or so newly calculated variables, called principle components. Principal components analysis (PCA) was performed using SAS JMP in 5.1 software.

5.3 Results.

5.3.1 GC-MS analysis of polar metabolites from *S. nodorum* infected wheat.

The aim of this experiment was to document the changes in primary metabolism during infection and sporulation of *S. nodorum* in wheat. Infected leaves to be used for a latent period assay were detached from the plant and mounted on benzimidazole agar to allow infection to progress. Triplicate SN15 infected and mock infected leaves were sampled. Infections were allowed to progress until 8 dpi, 10 dpi and 12 dpi (Figure 5.2). At 8 dpi, the leaves were mostly undamaged, with occasional small areas of necrosis caused by the infection. At 10 dpi, the fungus was rapidly spreading throughout the leaf, widespread chlorosis was apparent, and some pycnidia were visible. By 18 dpi, the leaf was mostly dead, the fungus had colonised almost all of it, and sporulation was widespread. By sampling at those times the process of sporulation could be correlated to metabolite abundance. Any metabolites that correlated strongly with sporulation provided leads for gene knockout studies to confirm genes that were required for sporulation.

Polar metabolites were extracted from the samples, derivatised, and analysed by GC-MS. A representative chromatogram from each treatment is shown in Figure 5.3. Chromatograms were analysed using AMDIS software to integrate the area of each peak and assign a MST match to the library of standards. All peaks that were integrated are shown in Table 5.1 with a retention time, identity, and average abundance. Each metabolite is also graphed in Figure 5.4. Ninety four peaks were detected in two of the three replicates. Sixty metabolites were assigned an identity, if a metabolite produced two peaks on the chromatogram the areas were summed to give a total response.

Figure 5.2

Figure 5.3

Table 5.1 A

Table 5.1 B

Figure 5.4 A

Figure 5.4 b

Figure 5.4 c

Figure 5.4 d

Figure 5.4 e

for that metabolite. Metabolite identities were grouped into several broad classifications including sugars (6), polyols (3), polyamines (2), amino acids (18), carboxylic acids (10), fatty acids (4) and sterols (2) leaving 35 unidentified (Table 5.2).

5.3.2 Metabolite distribution at time points.

The first analysis involved checking at which time point each metabolite was detected. This showed how diverse each growth stage was in terms of presence or absence of metabolites. The distribution of metabolites within the three SN15-infected time-points were shown as a Venn diagram in Figure 5.5. Most of the metabolites were detected in all three infected time-points. Six metabolites were only found at 12 dpi in SN15-infected samples. They were: beta-alanine, methionine, tyramine and unknowns at 30.2982 min, 36.4750 min, and 43.9808 min. Five metabolites were found only in 10 dpi and 12 dpi infected samples. They were: fumaric acid, maleic acid, trehalose, unknown – 32.6593, and unknown – 48.1439.

For comparison, the same procedure was performed on results for mock-infected samples (Figure 5.6). Similarly to the SN15 infected samples, most metabolites were found at all three time points. Seven metabolites were found only at 12 dpi in mock-infected samples. They were: fumaric acid, isocitric acid, maleic acid, trehalose, and three unknowns at 30.8177 min, 43.7743 min, 43.9808 min. Seven metabolites were found only in 10 dpi and 12 dpi mock-infected samples. They were: asparagine, 9,12,15-octadecatrienoic acid, unknown-30.3602, unknown-35.7046, unknown-46.7613, unknown-46.7892, and unknown-49.4135. Another comparison can be made using the presence of each metabolite in SN15 and/or mock infected samples (Figure 5.7). Seventy two metabolites were found in both infected and uninfected samples,

Table 5.2

Figure 5.5

Figure 5.6

Figure 5.7

which is approximately 80% of the total. Metabolites found in only SN15-infected samples were: beta-alanine, methionine, suberyl glycine, unknown-29.9914, unknown-30.2982, unknown-32.6593, and unknown-36.4750. Metabolites found only in the mock-infected samples were: isocitric acid, unknown-33.3700, unknown-43.7743, unknown-44.5212, unknown-46.7613, unknown-46.7892, and unknown-49.4135.

5.3.3 Hierarchical clustering of *in planta* metabolomics data.

Ward's minimum variance method of hierarchical clustering (JMP in 5.1, SAS) was applied to the observations for the two infection treatments and the three time-points (Ward, 1963). The samples clustered into six groups based on metabolite abundances, four of which correlated perfectly with the three time points and treatments used (Figure 5.8). The 8 dpi mock, 8 dpi SN15, 10 dpi SN15 and 12 dpi SN15 samples all grouped together. The remaining two groups had one sample assigned incorrectly in each, a 10 dpi mock was grouped with the 12 dpi mock and vice versa. The SN15 and mock infected samples at 8 dpi clustered into two similar groups, but the 10 dpi and 12 dpi SN15 infected samples formed two groups at the other end of the scale, showing that together they were quite similar. The 10 dpi SN15 samples appeared to be closer to the 12 dpi mock infected samples than the 12 dpi SN15 infected samples.

5.3.4 Hierarchical clustering of individual metabolite profiles.

In Figure 5.8, clustering showed that biological samples could be differentiated by their metabolite profiles. HCA was then used to find which metabolites have similar profiles during sporulation. Hierarchical cluster analysis was performed on the ratio of the metabolite abundance in SN15 infected to its abundance in mock infected samples. Metabolites with more than one peak were analysed as summed values and as independent values. The three replicates were averaged before the ratio was calculated,

Figure 5.8

giving three ratios per metabolite, at 8 dpi, 10 dpi and 12 dpi. In this way the dataset was simplified somewhat, but importantly, aberrations caused by variable abundance were ameliorated. Because of the huge changes in magnitude observed for some metabolites, the values were log10 transformed to allow clearer visualisation of the changes.

The cluster analysis dendrogram was used to partition the metabolites into eight groups (Figure 5.9). These groups formed on the basis of the magnitude of the ratio between SN15 and mock infected leaves and also on the different ratios at each time-point. Group 1 (41 members) and group 2 (26 members) accounted for the samples that changed little, generally with group 1 slightly down in SN15 infection and group 2 slightly up in SN15 infection. Groups 3 to 8 accounted for metabolites with larger changes, increasing in magnitude with each subsequent group.

Group 3 contained seven members that peaked at 10 dpi and were less abundant at 8 and 12 dpi, it was composed of five unknowns, maleic acid and stigmasterol. The eight members of group 4 showed a general decrease in abundance at 10 and 12 dpi. Group 4 consisted of putrescine, isocitric acid and six others of unknown identity. Group 5 had only tyrosine and an unknown in it, both present in lower amounts during the initial two time-points and equal amounts at day 12. Group 6 had seven metabolites all of which showed an increase in the SN15 infected samples at 8 and 12 dpi with a trough of sorts at 10 dpi. Metabolites in this group were: leucine, asparagine, suberyl glycine, glutamine and fructose. One of the two glucose peaks was the sole representative of group 7, it had a much larger amount in the SN15 samples at 8 and 10 dpi. Group 8 represented the metabolites with abundances that positively correlated strongly with

Figure 5.9 a

Figure 5.9 b

sporulation. They all had a large increase in abundance at 10 and 12 dpi in the SN15 infected leaves. Group 8 included glutamine, trehalose, mannitol, and two unknowns with retention times of 32.6593 and 48.1439 minutes. It showed the ideal pattern of increasing abundance as sporulation occurred coupled with large magnitude changes.

5.3.5 Principle components analysis of *in planta* metabolite profiles during sporulation.

Principle components analysis was performed on the *in planta* dataset. The principle components on correlations were calculated for the *in planta* dataset, the loading for each sample was recorded and two factors plotted on an XY graph. Factors one versus two are shown in Figure 5.10 A, factors three versus four are shown in Figure 5.10 B and factors five versus six are shown on Figure 5.10 C. Principle components each describe a percentage of the total variation in the data. In this case, principle component one (PC1) explained 27.2%, PC2 19.6 %, PC3 13.4 %, PC4 8.5 %, PC5 5.9 %, and PC6 4.5 %. Together, the first six components therefore explained a total of 79% of the total variation. Comparison of the principle components by samples, showed a similar result to the cluster analysis, in that each of the treatments could be distinguished on the basis of the variation in metabolite abundances. PC1 distinguished between 12 dpi SN15 infected from all other samples. PC2 did not clearly distinguish any of the treatments. PC3 describes difference between all SN15 infected samples and mock infected. PC4 differentiates infected SN15 8 dpi from all other samples. PCs 5 and 6 do not resolve any of the sample treatments. Figure 5.10 A shows that in a PC1 versus PC2 plot, each treatment groups quite well, with only the 10 dpi mock infected samples having a large spread. Plotting only PC3 and PC4 in Figure 5.10 B shows that even some of the minor components are still sufficient to resolve the treatments for SN15 infected at 8 dpi and 10 dpi. The PC5 versus PC6 plot (Figure 5.10 C) shows that they do not contain so

Figure 5.10

much useful information, as on the basis of those components none of the samples can be resolved from the others.

Once the components have been calculated, the individual contributions of each metabolite can be graphed to show which ones are the most important. Metabolite loadings for PC1, PC2, PC3 and PC4 are shown in figures 5.11 A, B, C and D, respectively. Component one is the most informative in regard to sporulation of SN15 *in planta*. As mentioned previously, PC1 clearly distinguishes the 12 dpi SN15 infected samples from all others. The 12 dpi SN15 samples have the most sporulation structures present and therefore make PC1 a good source of sporulation specific information. The most significant metabolites in PC1 were, in order of impact: malic acid, quinic acid, glutamine fumaric acid, mannitol, phosphate, beta-sitosterol, methionine, 2-ketoglutaric acid, alanine, glyceric acid, trehalose, octadecatrienoic acid, hexadecanoic acid, inositol, suberyl glycine and eight unknowns. The most significant loadings for PC2 were; isoleucine, lysine, threonine, phenylalanine, leucine, nonadecanoic acid, tyrosine, pyroglutamic acid, fructose, serine, aspartic acid, putrescine, valine, ornithine, tyramine, alanine, sucrose, glutamate (Figure 5.11 B). Interestingly, of the 50 metabolites shown, only four metabolites had a negative factor loading.

Components can be calculated so that individual metabolites can be plotted as PC1 and PC2 factor loadings on an X Y graph (Figure 5.12). The graph of all the detected metabolites showed all of the metabolites that increase in abundance during infection group together. These metabolites are individually labelled on the plot, and all have a general profile of increasing abundance in SN15 infected samples as infection progresses. They were, mannitol, trehalose, methionine, glutamine, and four unknowns

Figure 5.11 a

Figure 5.11 b

Figure 5.12

with retention times of 30.2982 min, 32.6593 min, 36.4750 min , 38.4362 min and 48.1439 min. To see if classes of metabolites behaved similarly during infection, metabolites were put into general classes and given distinct graph markers according to their class. The classes used were; sugars, sugar alcohols, amino acids, carboxylic acids, polyamines, sterols, and any remaining metabolites marked as black dots on the graph. The distribution of the classes was very even about the plot, which shows that general classes of metabolite, as a whole, were not co-regulated.

Correlations between different metabolite abundances indicate they may be co-regulated under the experimental conditions. Correlation coefficients were calculated for log transformed abundances of each metabolite detected (Table 5.3). A positive correlation coefficient above 0.9 was considered informative. These co-regulated metabolites are shown in Table 5.3. Amino acids were the most common group represented in the table, found in six of the eleven comparisons shown. Aspartic acid 3TMS and glutamic acid 3TMS had the highest correlation of 0.96. Tyramine and asparagine both correlated strongly with unknowns. Ribitol and nonadecanoic acid were both internal standards and are included in this table as a proof of principle. As both standards were added to the samples in an equal ratio they should correlated very well, as is the case here with a correlation of 0.94. Malic acid and quinic acid correlate well (0.94) as did mannitol and trehalose (0.92), these four metabolites have been noted previously in the HCA analysis (Section 5.3.1.2) as correlating with sporulation.

5.3.6 t-Test statistical analysis of metabolite abundances.

Mean values for each treatment were compared using a one-tailed t-test (EXCEL, MICROSOFT) to check the significance of any changes, and further checked by the more rigorous Tukey-Kramer test (JMP, SAS) for confirmation. Some metabolites

Table 5.3

were not detected in various treatments or time points making them unsuitable for statistical comparisons. In these cases, values slightly less than the detection threshold were substituted for missing values and procedures were repeated with close manual inspection of the results. Mean abundances in infected vs uninfected samples were compared at each time-point by t-test and showed there were 28 mean differences with a P value of less than 0.05 (Table 5.4). In addition to comparison of SN15-infected and mock-infected samples, SN15-infected samples were compared at each of the three time points (Table 5.4). These results were used to gauge relative differences in significance.

5.3.7 Tukey-Kramer statistical analysis of metabolite abundances.

The Tukey-Kramer method of significance was performed on the *in planta* dataset (Table 5.5), testing the significance of differences between SN15-infected samples over the three time points (Table 5.6). At an alpha value of 0.05, fifteen metabolites had significantly different means. The metabolites were grouped according to the overall profile of abundance. Threonic acid, beta-sitosterol and unknown-44.6951 had a significant difference between 8 dpi and 12 dpi mean abundances. Asparagine, fructose and glucose had a significant difference in mean abundance at 8 dpi and 10 dpi, and fructose and glucose had a significant difference in mean abundance between 8 dpi and 12 dpi.

Glycine had a significant difference between 10 dpi and 12 dpi. Glutamine and quinic acid had a significantly higher abundance at 12 dpi compared to 8 dpi. Six metabolites had a significantly higher abundance at 12 dpi compared to both 8 dpi and 10 dpi. They were, phosphate, malic acid, mannitol, and unknowns at 26.5705, 38.4362, 46.4977 minutes.

Table 5.4 a

Table 5.4 b

Table 5.4 c

Figure 5.4 d

Figure 5.5

Figure 5.5

Figure 5.5

Figure 5.5

Figure 5.6

Metabolites that had significant increases as sporulation occurred, in either the t-test analysis or the Tukey-Kramer analysis, were compiled into a list of "interesting" metabolites. In addition to the eight metabolites with significant changes named in the Tukey-Kramer analysis, another seven were considered important based on the t-test results, methionine, beta-alanine, trehalose, and three unknowns at 32.6593 min, 30.2982 min and 36.4750 min. The raw data for these metabolites was graphed in Figure 5.13.

These metabolites with significant changes were grouped according to their profiles, in this case corresponding to whether they were increasing or decreasing in abundance over time. The first group included those with steady state levels at 8 and 10 dpi and an increase in the SN15 12 dpi samples. Metabolites in this group are phosphate, and unknown-26.5705 (Figure 5.13 A). The second group includes those with a general increasing trend over time in the SN15 infected leaves (Figure 5.13 B). These metabolites steadily increased in abundance during infection, but did not in the mock infected samples. The four metabolites are quinic acid, malic acid, and two unknowns at 38.4362 min and 46.4977 min. The third group observed showed a clear difference between infected and uninfected samples and a large increase in abundance at 10 and 12 dpi (Figure 5.13 C). These metabolites were, mannitol, trehalose and an unknown at 32.6593 min.

There was also a group of metabolites that were only detected in the 12 dpi SN15 infected samples (Figure 5.13 D). As they were not found in any other time points, comparative statistics were not possible, but they were approximately an order of magnitude above the detection limit and were reproducible within those replicates.

Figure 5.13 a

Figure 5.13 b

The metabolites in group four were, beta-alanine, methionine and two unknowns at 30.2982 and 36.4750 min. The final group of metabolites were ones with a general decreasing trend (Figure 5.13E). Two metabolites fitted this description, fructose and beta-sitosterol. Fructose showed a reduction in both infected and uninfected samples but during infection the decrease was apparent by 10 dpi, while in the mock treated leaves the decrease was only seen at 12 dpi. Beta-sitosterol abundance gradually decreased over the time course in both infected and uninfected samples, but there was always less in the infected samples.

5.4 Discussion.

5.4.1 Aims

A metabolomics based approach to investigate sporulation of *S. nodorum* SN15 was undertaken. The metabolome was investigated during growth in the host, *Triticum aestivum*, before and during asexual sporulation. The main aim was to identify metabolites that were likely to be produced by *S. nodorum* during sporulation. These metabolites were then to provide a lead to genes required for sporulation. The experimental system involved two interacting organisms, analysed as one sample. Therefore, the true origin of each metabolite could never be conclusively stated. As such, cataloguing every metabolite was not the main outcome, rather the aim was to identify a set of metabolites strongly associated with sporulation.

5.4.2 Method development.

A method for the analysis of plant polar metabolites by GC-MS has been successfully used for the study of *S. nodorum* infected wheat. Time consuming optimisation was not required as GC-MS protocols are highly reproducible across different equipment. In

addition, the use of an established method allowed the utilisation of published MST libraries of common metabolites.

The delivery of sample to the gas chromatograph and the detection of the same metabolites by the mass spectrometer is highly reproducible, far more so than the preparation of the sample. Potential problems during sample preparation were numerous. The most confounding problem was enzymatic activity in biological samples. When a living organism is harvested, it is usually harshly removed from its growing state, resulting in tissue damage or other stresses. These stresses can alter the physiology of the sample very rapidly. Also, once a living sample is disrupted prior to metabolite extraction, the metabolites are exposed to enzymes that would normally be kept apart, and biological equilibria are altered. To minimise these two problems, tissues were snap-frozen in liquid nitrogen immediately after harvesting, and were disrupted after cooling in liquid nitrogen. Cryogenic temperatures minimise tissue stress responses, enzymatic activity, and non-enzymatic changes such as oxidation. The initial extraction was carried out at 70ºC to help inactivate enzymatic activity during the methanol-chloroform solvent extraction.

The amount of sample analysed must be precisely known, so that variation in sample size can be normalised. Metabolites were extracted from the sample with a series of solvents and involved a necessary loss of sample in order to ensure the purity of the extract. The concentration of derivatised metabolites was affected by a further two variables, the volumes dispensed and the evaporation of the highly volatile reagents. Normalisation of sample amount carried through the procedure was therefore a critical part of the analysis. Sample loss or concentration was corrected by the addition of an

internal standard not found in the biological system being analysed. Ribitol is often used for this procedure and was suitable as it has not been detected in either *S. nodorum* SN15 or wheat cv. Amery. A defined amount of ribitol was added to the tissue immediately after disruption, the amount carried through to detection was proportional to the amount of dilution or concentration of the sample during the entire process.

The criteria for peak identification were deliberately very strict. If peaks were not identified in two of the three replicate samples, they were disregarded. A metabolite peak had to match the retention time and have a close mass spectral tag (MST) match to an authentic standard before being identified. Because the metabolomics screen was being used to identify genes related to sporulation, and to subsequently further investigate these genes by gene mutagenesis studies, the consequences of an incorrectly identified metabolite could be disastrous. Only metabolites identified with certainty could be used for further work. For this reason, unknowns are recorded but may not be mentioned during the discussion.

5.4.3 GC-MS analysis of *S. nodorum* infected wheat.

SN15 infected wheat plants were sampled at 8 dpi, 10 dpi and 12 dpi (Figure 5.1). Metabolites were extracted from the entire infected leaf, and analysed by GC-MS. Ninety four different peaks were detected (Figure 5.2), and of those 60 were assigned an identity (Table 5.1, Figure 5.3). This is an acceptable level of resolution for a GC-MS study of this kind. In Roessner (2000), where the basic GC-MS method was first published, approximately 150 peaks were resolved and 77 metabolites were identified. The number of peaks resolved was limited by a high degree of variation between biological replicates, leading to some metabolites dropping below the threshold for detection. The GC column used for separation was an Agilent HP50+ column, which

contains a high-bleed stationary phase. A low-bleed (5% phenyl 95% dimethylpolysiloxane) GC column such as the Varian FactorFour column would produce less background signal and could increase the number of low abundance peaks resolved.

A range of metabolite classes were identified: sugars, polyols, polyamines, amino acids, carboxylic acids, fatty acids and sterols (Table 5.2). This was the expected range, as the extraction procedure was designed to isolate polar metabolites and GC-MS is best suited to the analysis of small metabolites that are easily volatilised after trimethylsilylation. Complex secondary metabolites would be too unstable or large to be detected by GC-MS.

Most identifications were relatively straightforward, with both a retention time match and a mass spectral tag match. However, the peak at 22.5761 was identified as either xylitol or arabitol, which was because the retention times for both metabolites were very close and could not be resolved at the concentrations detected *in planta*. Additionally, the mass spectra for the two polyols were indistinguishable, as they are isomers. Authentic standards for xylitol and arabitol have both been identified as xylitol by mass spectral matching. The peak was named xylitol/arabitol in recognition that it was undoubtedly some combination of one or both polyols, but could not be resolved any further.

5.4.4 Sporulation associated metabolites

The aim of studying the metabolome of *S. nodorum* during infection was to find out which metabolites were positively associated with sporulation. Two metabolites stood out among all of the analyses, mannitol and trehalose. Mannitol abundance increased

30-fold in SN15-infected samples by 12 dpi (Figure 5.13 C), while trehalose was only detected at 10 dpi and 12 dpi in SN15-infected samples, increasing in both (Figure 5.13 C). After both HCA and PCA analyses mannitol and trehalose grouped together as metabolites that increased in abundance in SN15-infected samples as sporulation occurred (Figures 5.8, 510, and 5.11). The abundance of mannitol and trehalose was strongly correlated with each other, with a correlation coefficient among the 10 highest observed. This suggests that they may be co-regulated at the transcription or enzymatic level (Table 5.3). The increases in observed abundance were significantly different for mannitol. A t-test analysis of mannitol mean abundances in SN15-infected and mock-infected samples showed that the means were different with P values of 0.0119 at 10 dpi and 0.0098 at 12 dpi, respectively. Trehalose was not detected at 8 dpi or 10 dpi in mock-infected samples, preventing the use of t-tests for those samples. It was detected in both 12 dpi samples, the difference was significant at a P value of 0.0586, which is not as significant as the mannitol difference.

The more rigorous Tukey-Kramer significance test was also used to analyse the mean abundances of each metabolite in the SN15-infected samples. Mannitol was significantly more abundant at 12 dpi than at 8 or 10 dpi. This meant that mannitol was (a) positively associated with sporulation, (b) one of the most abundant metabolites at 12 dpi and (c) was a statistically robust observation. The changes to trehalose abundance were not as statistically valid as those for mannitol, but it was still considered a key metabolite correlated with sporulation.

Mannitol and trehalose were often grouped with other metabolites after both HCA and PCA analyses, including glutamine, methionine, malic acid, quinic acid, fumaric acid.

While glutamine, quinic acid and malic acid changes were significantly different under both statistical tests, they cannot be attributed to either fungal or plant origin. They have been shown to change in abundance as infection occurs, but they cannot be linked with sporulation in this experimental situation. For example, they may be a result of wheat senescence which occurs as infection progresses. The biological significance of mannitol and trehalose in relation to sporulation of fungi was much greater.

5.4.4.1 The biological significance of mannitol and trehalose.

Both trehalose and mannitol are commonly found in fungi (Lewis and Smith, 1967). Neither metabolite has been reported to be found in wheat (Abebe *et al.*, 2003; Solomon *et al.*, 2006b). Mannitol is an acyclic six carbon sugar alcohol, derived directly from fructose (via mannitol dehydrogenase, EC 1.1.1.138), or via fructose 6-phosphate and mannitol 1-phosphate (mannitol 1-phosphate dehydrogenase, EC 1.1.1.17 and D-mannitol-1-phosphate phosphatase EC 3.1.3.22) (Boonsaeng *et al.*, 1976; Trail and Xu, 2002).

Mannitol has been suggested to play various diverse biological roles in fungi. A key discovery in relation to this study was that mannitol is required for asexual sporulation in *S. nodorum* SN15 (Solomon *et al.*, 2005a; Solomon *et al.*, 2006e). The reason for this dependence is not yet known, but is probably due to a combination of roles already reported in other fungi. Mannitol has been suggested as a major store of carbon in *Aspergillus niger*, especially in spores (Witteveen and Visser, 1995), however in *S. nodorum*, lipids are thought to be more important for early spore germination and growth (Solomon *et al.*, 2004a). Oxidative stress is a common problem for plant pathogens, and mannitol has been suggested to ameliorate this stress in pathogenic fungi , including *Alternaria alternata* (Jennings *et al.*, 1998; Jennings *et al.*, 2002),

Cyptococcus neoformans (Chaturvedi *et al.*, 1996) *Uromyces fabae* (Voegele *et al.*, 2005), in the opportunistic pathogen *Aspergillus niger* (Ruijter *et al.*, 2003), and the bakers yeast *Saccharomyces cerevisiae* (Chaturvedi *et al.*, 1997). The effect is thought to be a result of mannitol quenching reactive oxygen species. Mannitol has also been suggested to reduce salt and heat stress (Chaturvedi *et al.*, 1997; Managbanag and Torzilli, 2002; Ruijter *et al.*, 2003; Stoop and Mooibroek, 1998).

Trehalose is a non-reducing disaccharide comprised of two glucose monomers joined by an alpha-1 alpha-1 linkage. Trehalose is often found in fungi along with sugar alcohols and glycogen, and in particularly high levels in reproductive structures (Thevelein, 1984). In a similar fashion to mannitol, trehalose has many diverse roles in fungi, for example generation of the osmotic potential required for appressorium turgor in *M. grisea* (Foster *et al.*, 2003), and combating heat and oxidative stress in *Aspergillus niger* (Fillinger *et al.*, 2001). These roles have not, however, been investigated in *S. nodorum*. A role in generation turgor pressure may be possible, *S. nodorum* has been variously reported to produce or not produce appressoria during direct penetration of the plant cell wall (Baker and Smith, 1978; O'Reilly and Downes, 1986; Shipton *et al.*, 1971; Solomon *et al.*, 2006f). Its use as a stress response metabolite or storage carbohydrate is a strong possibility. A more in-depth investigation of trehalose is presented in the chapter 7 introduction.

An unexpected result from the study was that mannitol and trehalose were detected in mock infected samples (26.1804 minutes, 40.4500 minutes Table 5.1), albeit at a much lower abundance than in SN15-infected samples. At 8 dpi, equally small amounts were detected in mock and SN15-infected samples. At 10 dpi, the SN15-infected samples

had 10-fold more mannitol than in the mock-infected samples, and by 12 dpi there was almost 50-fold more mannitol in SN15-infected samples. The small level amounts of mannitol in mock-infected leaves was probably due to low-level colonisation of the leaf surface by opportunistic microbes. The latent period assay leaves used for the samples were not grown in sterile conditions. The wheat plants were 2-weeks-old before being inoculated with SN15 spores and were a further 8 to 12 days old before being harvested. During this time it is quite possible environmental microbes could grow on the leaf surface without causing lesions or visible tissue damage.

5.4.5 Metabolite distribution at time points.

The presence or absence of each metabolite was recorded for each time point and infection state. It showed that most metabolites were found at all three time points in both SN15 infected (Figure 5.5) and mock infected (Figure 5.6) samples. Most metabolites were found in both infected and uninfected samples, but seven were only found in SN15 samples and another seven were only found in Mock samples (Figure 5.7). For the SN15-infected and mock-infected samples, xylitol/arabitol and ribose was only found in 8 dpi samples, both at quite low abundances. This could not be attributed to *S. nodorum* infection as it was present in the mock-infected sample at a similarly low abundance.

Identified metabolites only present at 12 dpi SN15-infected samples were: beta-alanine, methionine and tyrosine. It is interesting that three amino acids would be represented at this time point, although they are not closely linked in a single biosynthetic pathway. Beta-alanine and methionine were only detected in SN15-infected samples. Perhaps

they are key breakdown products of senescing plant tissue or a major constituent of fungal biomass.

Trehalose, maleic acid and fumaric acid were only found at 10 dpi and 12 dpi in SN15-infected samples. Trehalose and fumaric acid both were present at quite high abundances at these time points suggesting a more rapid and definite increase late in infection. Fumaric acid is a carboxylic acid member of the citric acid cycle, found in plants and fungi.

5.4.6 HCA of *in planta* metabolite changes.

Hierarchical cluster analysis (HCA) was used to cluster the data by metabolite abundances into manageable groups. Clusters were formed among samples that were most similar. If metabolite profiling was sufficient to describe the changes occurring during sporulation *in planta*, HCA should have been able to correctly cluster sample replicates based solely on their metabolite abundances. HCA was performed on the ratio of SN15-infected to mock-infected metabolite abundances. When raw values were used for clustering, the result was skewed toward clustering samples with similar abundances rather than by their change in abundance over time. When ratios were used, the average abundance was normalised between metabolites, and the fold change was most important.

Figure 5.7 shows how the 18 samples clustered after HCA. The 8 dpi mock, 8 dpi SN15, 10 dpi SN15 and 12 dpi SN15 infected samples were all clustered with their own replicates. The 10 dpi and 12 dpi mock infected samples swapped one sample between the two groups. This is not unexpected as the mock-infected samples did not senesce during the experiment, and physically looked very similar across the three time points.

Metabolically, they would be expected to be similar, and hence difficult to resolve. The three time points from SN15-infected leaves all looked very different, with rapid plant chlorosis and necrosis occurring, and fungal structures becoming visible at 10 dpi and 12 dpi. These physical differences were mirrored by metabolic differences, sufficient to distinguish the time points. This experiment showed that the metabolome of SN15 and wheat was changing rapidly, and could potentially account for the physical differences observed.

HCA was also used to group the 97 different metabolites according to their abundance ratio of SN15-infected divided by mock-infected abundance, over the three time points (Figure 5.9). Eight groups were formed.

HCA groups 1 and 2 contained metabolites that did not change markedly (vary from 0.5) over the three time points. These two groups contained 67 metabolites. Group 3 contained seven metabolites, which all peaked at 10 dpi. Given the physiological changes to the samples, which were progressive over the time course, is difficult to explain why these metabolites would peak in the 10 dpi samples. The two identified members of group 3 were maleic acid and stigmasterol. Group 4 contained eight metabolites that were usually found in lower amounts in SN15-Infected than in mock infected leaves. These metabolites, putrescine, isocitric acid and six unknowns, may represent metabolites that are rapidly consumed during infection, especially after 12 dpi. They are unlikely to be fungal derived, as fungal biomass increased dramatically by 12 dpi. These metabolites could be candidates for sporulation-repression treatments, if their abundance is kept at a high level during infection, by external application for example, they might repress sporulation.

The two unidentified metabolites in group 8 are still of interest, especially if they can be conclusively identified in the future. They have mass spectral matches of reasonable quality, but no corresponding retention-time matches could be found. Unknown – 32.6593 matches phytol (1TMS) with a quality score of 88/100. There was not a phytol standard with retention time matching for the method used. It is quite possible that the unknown-32.6593 is phytol, as phytol has a 16-carbon alcohol backbone with three methyl groups spaced along the backbone. The length and functional group composition of phytol is very similar to hexadecanoic acid, which is known to elute less than 2 minutes prior to the unknown. The only way to conclusively check this hypothesis would be to run a phytol standard with the same GC-MS method. The source of phytol would most likely to be chlorophyll, which can be broken down into phytol, via chlorophyllase (EC 3.1.1.14) a plant enzyme. This activity has been documented in senescing plant leaves (Pruzinska *et al.*, 2005).

Unknown – 48.1439 matches sucrose (8TMS) with a quality score of 98/100. The retention time of this metabolite did not match a standard and could not be confirmed as identified. It is definitely not sucrose, as that has a retention time of 39.01 minutes. It is likely to be a trisaccharide, as it elutes much later than all the disaccharides, and is closer to the elution time of raffinose, a known trisaccharide. Mass spectral fragmentation patterns from di and tri saccharides are quite similar and so could be easily confused. Analysis of a suite of trisaccharide standards may help identify this unknown metabolite in future experiments.

5.4.7 PCA of *in planta* metabolomic changes.

PCA was used to analyse the *in planta* sporulation metabolome dataset. The dataset comprised 96 different metabolites, and hence 96 variables. PCA reduced the complexity down to 6 major components (variables) that together described 79% of the total variation (Figure 5.10). A plot of PC1 versus PC2 was able to resolve each of the three SN15-infected time points. The plot could also resolve 8 dpi mock from 10 dpi and 12 dpi mock samples. However, 10 and 12 dpi mock samples could not be distinguished using PC1 and PC2. PCs 3 and 4 could 8 dpi SN15, 10 dpi SN15 from other samples. PCs 5 and 6 did not contain enough information to distinguish the sample types. PC1 and PC3 were the components that best separated the sample types.

Each principal component was broken down into the factor loadings for each metabolite, revealing the contribution made toward the component. The top 25 factor loadings for PC1, PC2, PC3, and PC4 were calculated, and were shown in Figure 5.11. PC1 captured the most variation, and metabolites that contribute the most to it tend to contain large increases in abundance in SN15-infected samples at 10 and 12 dpi. There were four metabolites in the top 25 most influential PC1 graph that had negative factor loadings, which indicated they acted in the opposite manner to the majority of metabolites in the graph. These negative-loading metabolites tended to be less abundant in SN15-infected samples, and decreased in abundance at 10 dpi and 12 dpi.

Key influential metabolites from on the PCA results were malic acid, quinic acid, glutamine, fumaric acid, mannitol and trehalose. Malic acid and fumaric acid are both members of the tricarboxylic acid (TCA) cycle, part of primary metabolism and both are substrates of fumarate hydratase (EC 4.2.1.2), this link helps to explain why both have

similar abundance profiles. Three amino acids are present in the top 25 metabolites for PC1, glutamine, methionine and alanine. Also, quinate is involved in the shikamate pathway, near to tyrosine and tryptophan biosynthesis. An increase in free amino acids in SN15-infected samples may indicate proteolysis or cell lysis is occurring, liberating amino acids. However, it is more probable that the increase of these particular amino acids represents the movement of stored metabolites to these amino acids, perhaps as *S. nodorum* degrades the host tissue.

Chapter 6

Metabolomics of *in vitro* sporulation.

6.1 Introduction

As mentioned previously, there has not been any published attempt to analyse the metabolome of *S. nodorum*. Chapter 5 investigated the SN15-wheat infection metabolome. The *in planta* study was limited by the inability to determine the origin of almost all the metabolites detected. Here, the metabolome of *S. nodorum* was investigated during the asexual life-cycle in pure culture to reveal key metabolic changes that occured before and during sporulation. Metabolites identified from *in vitro* grown cultures are highly informative, as they are derived entirely from fungal growth, in comparison to the *in planta* samples that contains both plant and fungal metabolites. The SN15 metabolome when grown in pure culture was analysed using GC-MS. For an in depth review of metabolomics, refer to chapter 5.

6.2 Materials and methods

6.2.1 Growth of SN15 *in vitro* cultures for GC-MS analysis.

SN15 pycnidiospores (1 x 10^6/plate) were spread on the surface of solid minimal media plates. Five plates were prepared per sample. Three replicate samples were prepared per time point. Plates were dried, sealed, and wrapped in aluminium foil to prevent exposure to light. Cultures were incubated at 20ºC and samples were harvested at 4 dpi, 11 dpi and 18 dpi.

6.2.2 Extraction of metabolites for GC-MS analysis.

Mycelia was harvested (approximately 50 to 100 mg fresh weight) from the surface of the plate by gentle scraping with a new scalpel blade (curved blade). Mycelia was snap frozen in liquid nitrogen and freeze-dried overnight in a Maxi Dry Lyo (Heto Holten, Allerød, Denmark) freeze-drier and weighed. Dried tissue was cooled in liquid nitrogen and disrupted for 1 minute at 30 Hz using a ball-mill (Retsch GmbH & Co. KG, Haan, Germany). Metabolites were extracted from disrupted tissue as described previously.

6.2.3 GC-MS analysis.

6.2.3.1 Retention-time locking

The GC-MS procedure was as previously described in chapter 5, with the following modification: the helium carrier-gas flow-rate was retention-time locked to elute mannitol at 24.30 minutes. This allowed direct comparison of retention times to authentic standards in published mass spectral libraries. The retention times for metabolites in this chapter are different to those in chapter five "metabolomics of *in planta* sporulation".

6.2.3.2 Analysis of saturated metabolite extracts

Metabolite extracts were derivatised as described previously, with the following modification. A 1 μL aliquot of derivatised metabolites was diluted 50-fold in MSTFA and sealed in a second GC-MS vial. Diluted and undiluted samples were analysed as described previously. The dilution factor and sample size were normalised for by dividing the metabolite peak area by the ribitol internal standard area and by the tissue weight. Samples were diluted in MSTFA as the TMS donating reagent must be in excess for the metabolites to remain derivatised.

6.2.4 Principal components analysis

6.2.4.1 Varimax rotation of components.

Principal components analysis factor loadings were either analysed as original factor loadings or as varimax rotated factor loadings. Factors were rotated using the varimax rotation function in JMP in 5.1 software, SAS. Varimax rotation increased the observable variation for each principal component, and hence made tabulated values easier to interpret.

6.2.5 Metabolic pathway data mapping

Metabolite abundance data was mapped to primary metabolic pathways using VANTED software (Junker *et al.*, 2006; Klukas *et al.*, 2006). VANTED stands for Visualization and Analysis of Networks containing Experimental Data. Data was imported into VANTED and mapped onto a custom pathway, drawn to best represent the available data. Biochemical pathway enzymatic steps were produced by reference to data on the KEGG website (www.genome.jp/kegg/) and a biochemistry textbook (Kanehisa and Goto, 2000; Stryer, 1996).

6.3 Results

6.3.1 A method to capture the entire metabolome range by GC-MS.

The *in vitro* grown fungal metabolome has a wider dynamic range of metabolite concentrations than *in planta* infected tissue. The wide range of metabolite concentrations was found to be beyond the capabilities of the GC-MS system (data not shown). Diluted and undiluted samples of *in vitro* grown SN15 were analysed to determine if saturating metabolites could be correctly resolved when analysed in two runs of differing concentration. Metabolites were extracted from SN15 grown, in triplicate, on solid minimal media at 11 dpi and separated by GC-MS. Samples were run as 1:1 and 1:50 dilutions, chromatograms of a problematic region is shown in Figure 6.1A. The change in the peak shape and retention time of mannitol-TMS in a saturated sample is characterised by asymmetric peak profiles. Analysis of the amount of mannitol in 11 dpi SN15 cultures showed that without dilution the amount of mannitol was grossly underestimated (Figure 6.1 B). The normalised area for mannitol was calculated from a sample that had overloaded the column, but at 1:50 dilution was correctly resolved. The normalised area of mannitol from the undiluted sample was 5.10×10^{-2} mg^{-1}, while from the 1:50 diluted sample it was 3.88 mg^{-1}, approximately 100-fold more than the amount from a saturated chromatogram..

The areas (which are corrected for dilution) show that the undiluted sample produced a value that was 1.3 % of the amount calculated from the diluted sample. In comparison, analysis of a less abundant, non-saturating metabolite, glycerol, from the same samples showed little difference in normalised area between the two samples. The normalised area of glycerol from the undiluted sample was 4.27×10^{-3} mg^{-1} and from the diluted

Figure 6.1

sample it was $5.61x10^{-3}$ mg^{-1}. The glycerol abundance calculated from the undiluted sample was 76 % of that calculated from the diluted sample, and the two values were not significantly different by t-test (P = 0.13).

6.3.2 The metabolome of *S. nodorum* during sporulation *in vitro*.

The metabolome of *S. nodorum* SN15 during sporulation was investigated by GC-MS. SN15 was grown on solid MM for 18 days and sampled at three time points, two of which are shown in Figure 6.2. Samples were taken at 4 dpi, 11 dpi and 18 dpi. After 4 days growth, the colony was entirely composed of colourless hyphae, by 11 days growth the colony has begun form pycnidia in sparse patches (data not shown) and by 18 dpi the colony was fully sporulating. By sampling at these times, any metabolites that correlated strongly with sporulation could identify candidates for gene knockout studies to confirm sporulation specific requirements.

Polar metabolites were extracted from harvested fungal tissue and derivatised, then analysed using GC-MS. In order to capture all metabolites within the linear range of the GC-MS, dilutions of each sample were analysed in parallel. A representative chromatogram from each time-point is shown in Figure 6.3. Chromatograms were subjected to peak integration and deconvolution, which reliably detected 147 different peaks amongst all samples. Metabolites were assigned to peaks on the basis of their retention time (RT) and their mass spectral tag (MST). Fifty eight metabolites were formally identified. Table 6.1 lists the normalised abundances for each metabolite as an average and standard deviation. Each metabolite was graphed for reference in Figure 6.4, individual graphs are labelled by the retention time of the metabolite. The metabolites were also graphed as a pie chart to reveal the main components of each developmental stage (Figure 6.5). At 4 dpi, the main metabolites (ranked by normalised

Figure 6.2

Figure 6.3

Table 6.1 a

Table 6.1 b

Table 6.1 c

Figure 6.4 a

Figure 6.4 b

Figure 6.4 c

Figure 6.4 d

Figure 6.4 e

Figure 6.5

peak area) were; glucose, fructose, mannitol, unknown-26.6841 and galactose. At 11 dpi, the main metabolites were; mannitol, fructose, unknown-24.4530, glucose and xylitol/arabitol. At 18 dpi, the main metabolites were; mannitol, xylitol/arabitol, unknown-24.4530, trehalose and phosphoric acid. The shifts in the metabolome of the cell was mostly a change from large amounts of glucose and fructose at 4 dpi, to large amounts of mannitol and less fructose and glucose at 11 dpi and 18 dpi. At 11 dpi and 18 dpi, mannitol accounted for almost three quarters of the total normalised peak area.

Metabolite identities were grouped into a number of broad classifications including amino acids (18 metabolites), sugars (9), carboxylic acids (9), polyols (8), fatty acids (4) leaving 35 unidentified metabolites (Table 6.2).

An analysis was performed to check at which time point each metabolite was detected. The distribution of metabolites within the three time-points are shown as a Venn diagram in Figure 6.6. Among the metabolites solely detected at 18 dpi, only isocitric acid and raffinose could be identified. Those present at 11 and 18 dpi include seven amino acids (asparagine, beta-alanine, isoleucine, methionine, proline, tryptophan and valine), two fatty acids (9,12-octadecadienoic acid and 9,12,15-octadecatrienoic acid) two sugars (maltose and trehalose) and 2-aminoadipic acid, citric acid, ononitol, nicotinic acid, glyceric acid 3-phosphate and 25 unknowns.

6.3.3 Hierarchical cluster analysis of metabolite profiles.

Hierarchical cluster analysis (HCA) was performed on the *in vitro* dataset as described previously. HCA was used to identify groups of samples that behaved similarly. The individual samples clustered into three main groups based on metabolite abundances, which matched the three time points sampled (Figure 6.7). The dendrogram showed

Table 6.2

Figure 6.6

Figure 6.7

that the metabolome sampled at each time-point was reproducible across biological replicates and remained distinct from the other two time-points. Of the three samples, the 11 dpi and 18 dpi samples were the more closely related, and correlated with the presence of sporulation structures in those two samples.

In addition to clustering samples based on their whole metabolome, individual metabolites could be clustered on the basis of their abundance over the three time-points (Figure 6.8). HCA was performed as before but using log10 transformed abundances. The resultant dendrogram was used to split the metabolites into ten groups, which approximated different abundance profiles. The abundance profile of each metabolite was described by assigning each of the three time-points an abundance level, low, medium or high.

The profiles observed were; medium-low-medium (group 1), low-low-medium (group 2), low-medium-low (group 3), medium-low-low (group 4), low-medium-medium (group 5), low-high-high (group 6), high-high-high (groups 7, 8), med-med-med (group 9) and high-high-low (group 10). The clusters are not 'perfectly' assigned as some metabolites showed unexpected placement. For example, pyruvate and sorbitol are both found at 4 and 11 dpi and absent at 18 dpi. They share a similar pattern of decreasing abundance over time, and are clustered into two different clusters (pyruvate in group 3 and sorbitol in group 10). The pattern of abundance was correlated with the onset of sporulation in the samples, and those which correlated especially strongly were chosen as sporulation-associated metabolites.

Figure 6.8 a

Figure 6.8 b

Key sporulation-associated clusters were; group 2, group 5, and group 6. Metabolites within group 2 were: isocitric acid, ononitol, raffinose and 17 other unidentified metabolites. Metabolites within group 5 were: tryptophan, beta-alanine, 2-aminoadipic acid, 3-phosphoglycerate, methionine, valine, isoleucine, asparagine, 9,12-octadecadienoic acid, 9,12,15-octadecatrienoic acid and 19 other unidentified metabolites. Metabolites within group 6 were: citric acid, trehalose and two other unidentified metabolites. Group 4 and group 10 had a negative correlation with sporulation. Group 4 contained sucrose, arginine and 13 unknowns. Group 10 contained erythritol, glutamine, sorbitol and two unknowns.

6.3.4 Principle components analysis of *in vitro* metabolite profiles during sporulation.

Principle components analysis (PCA) was performed on the *in vitro* dataset as previously described. PCA provided an additional method to classify metabolites based on their abundance. The principle components on correlations were calculated for the *in vitro* dataset, the loading for each sample was recorded and two principle components (PCs) were each plotted on an XY graph (Figure 6.9). PC1 described 52.3 % of the total variation, PC2 described 21.4 %, PC3 described 7.6 %, PC4 described 4.8 %, PC5 described 3.7 % and PC6 described 2.8 %. The contribution of each PC was more clearly shown in the XY graph. PC1 resolved the 4 dpi samples from the 11 and 18 dpi samples and PC2 separated each of the three time-points from each other. When PC1 and PC2 were plotted together, the three time-points were clearly differentiated. Figure 6.9 B and C showed that none of the minor components could resolve the different time-points when plotted alone or in combination. Since PC1 and PC2 could account for most of the differences between the three physiological states of sporulation, the individual metabolite factor loadings for each PC were reported. Because the

Figure 6.9

percentage of the total variation described by PC3, 4, 5 and 6 was very small, the contribution of each metabolite was not presented.

The factor loading for each metabolite within PC1 and PC2 is listed in Figure 6.10 A. Factor loadings were rotated using the varimax method to give the maximum variance per component. This made the numerical differences larger when presented in a tabulated form. HCA was used to sort the rotated factor loadings for each metabolite into clusters of based on their position on the bi-plot of PC1 versus PC2 (Figure 6.10 B). This broke up the metabolites into manageable and informative groups.

Metabolites that had a positive influence on PC1 were found in groups 4, 3, 7 and 2. Group 4 and group 3 had far larger factor loadings and hence greater influence than group 7 and group 2. As mentioned previously, Figure 6.9 showed that PC1 distinguished sporulating samples (11 dpi and 18 dpi) from non-sporulating samples (4 dpi), and PC2 distinguished the 11 dpi samples from the 4 and 18 dpi samples. Groups 4 and 3 contained metabolites that were positively correlated with sporulation. Group 3 is the largest group and contained metabolites with a strong positive contribution to PC1 and a slightly negative contribution to PC2. It contained the amino acids leucine, isoleucine, valine, threonine, beta-alanine, aspartic acid, asparagine, glutamate, phenylalanine, tryptophan, tyrosine and proline. Trehalose was the only sugar present. Carboxylic acids were also represented with fumaric acid, malic acid, citric acid and 3-phosphoglycerate all present. The three fatty acids in the group were: octadecanoic acid, 9,12-octadecadienoic acid and 9,12,15-octadecatrienoic acid.

Figure 6.10 a

Figure 6.10 b

Group 4 was smaller than group three and contained metabolites with a strong positive contribution to PC1 and a slightly positive contribution to PC2. Three amino acids were found in group 4, serine, glycine and methionine. An intermediate in lysine biosynthesis, 2-aminoadipate, was also found. Phosphate was in this group along with succinic acid. Mannitol was one of the largest contributors to PC1 in group 4, along with unknowns at 37.8096 min and 37.4810 min. The unknown-38.8096 had a moderate MST match with UDP glucose and unknown-37.4810 had a poor quality MST match to a disaccharide.

In contrast to groups 3 and 4, groups 5 and 6 had a negative contribution toward PC1. These metabolites were negatively correlated with colony age and pycnidia production. Group 5 contained eight metabolites: two aminoacids (lysine and alanine), glucose, galactose, pyruvate, glycerol and two unknowns. Group 6 contained 19 metabolites, mostly composed of 14 unknowns. Sucrose was the only identified sugar, and the remaining four metabolites were glutamine, pyroglutamate, homoglutamine and arginine.

The individual metabolite factor loadings for PC1 and PC2 were plotted in the same way factor loadings for entire samples had been plotted (Figure 6.11). The metabolites here related to the summed sample coordinates plotted in figure 6.9. Metabolites that were located in the same region of the graph as, for example, the 18 dpi samples were the metabolites that characterised that time-point. Regions that represented metabolites present only at 4 dpi, 11 dpi, 18 dpi, 4 and 11 dpi, 11 and 18 dpi and 4 and 18 dpi were shaded in grey and labelled. This showed that aminoacids, sugar alcohols and carboxylic acids were evenly distributed across the plot. Fatty acids were clumped in

Figure 6.11

one region, which was associated with high abundance at 11 and 18 dpi. Trehalose and mannitol also resided in the 11 dpi and 18 dpi areas. Most metabolites changed according to the time-point sampled, as there were almost no metabolites located at the centre of the graph. Several carboxylic acids were found in the 11 dpi and 18 dpi regions; citric acid, malic acid, fumaric acid, succinic acid and isocitric acid. All of the fatty acids identified were located within or close to the 11&18 dpi region. The region corresponding to presence mostly at 4 dpi was smaller than the 18 or 11 dpi regions. It contained metabolites, sucrose, arginine, pyroglutamic acid, and many unknowns. Glucose and glycerol was located near by to the 4 dpi region.

6.3.5 Statistical analysis of *in vitro* metabolite abundances.

Hierarchical cluster analysis and principle components analysis revealed many different metabolites whose abundance correlated with each other and the three growth stages sampled. To validate these observations the statistical significance of abundance differences had to be calculated.

The significance of mean values for metabolites at each time point were compared to the other two time points using a one-tailed t-test (EXCEL, MICROSOFT), and the Tukey-Kramer test (JMP, SAS), as described previously. The results of the t-test are shown in Table 6.3. Some metabolites were not detected in various treatments or time points making them unsuitable for statistical comparisons. In these cases, values slightly less than the detection threshold were substituted for missing values and the Tukey-Kramer procedure was repeated with close manual inspection of the results. The results of the t-tests showed that 99 of the comparisons had significantly different means ($P<0.05$). These could be broken down into each comparison with 38 from the 4 dpi versus 11 dpi

Table 6.3 a

Table 6.3 b

Table 6.3 c

Table 6.3 d

Table 6.4 a

comparison, 27 from the 11 dpi versus 18 dpi comparison, and 34 from the 4 dpi versus 18 dpi comparison were significantly different.

All means for detectable metabolites were compared using the Tukey-Kramer HSD test and the results for those with significant differences are shown in Table 6.4. Each metabolite was also graphed in Figure 6.12. The metabolites were grouped on the basis of their overall abundance profile; increasing, decreasing, or peaking over the time course. Six different groups were formed, four of which matched a profile of increasing abundance as sporulation occurs. The Tukey-Kramer test can be used to assign levels to each sample, in this case letters. A is greater then B, and B is greater than C. If groups shared a letter then they were not significantly different.

The first group matched a profile of C, B, A for the three time points 4 dpi, 11 dpi and 18 dpi (Figure 6.12 A). These metabolites had significantly different abundances for each time point, each one larger than the previous. The members of this group were, beta-alanine, fumaric acid, glyceric acid 3-phosphate, octadecanoic acid, trehalose, and two unknowns at 31.3499 min and 34.4377 min. The second group contained metabolites where all the means were significantly different and the abundances peaked at 11 dpi then droped at 18 dpi (Figure 6.12 B). The members were, 2-aminoadipic acid, 9,12-octadecadienoic acid, mannitol and succinic acid. The third group of metabolites contained tyrosine and three unknowns at 25.1345 min, 26.8577 min and 27.5524 min (Figure 6.12 C). They all showed a significantly larger mean abundance at 18 dpi compared to 4 dpi. The three unknowns also had a significantly larger 11 dpi abundance than the 4 dpi abundance.

Figure 6.12 a

Figure 6.12 b

Figure 6.12 c

Figure 6.12 d

Group four contained three metabolites with significant differences between the 4 dpi and 11 dpi means and between the 18 dpi and 11 dpi means (Figure 6.12 D). It included the metabolites fructose 6-phosphate, glucose 6-phosphate and unknown–37.8096. Group five contained five metabolites, malic acid, phenylalanine, serine, unknown–17.5396, and unknown–45.5899 (Figure 6.12 E). These metabolites had increased abundance at 11 dpi and 18 dpi that was significantly different to the amount at 4 dpi. The remaining metabolites were put into group six, which contained all those with a large average abundance at 4 dpi that then decreased at 11 and 18 dpi (Figure 6.12 F). These metabolites all had significant differences between the 4 dpi and 18 dpi time points. The group included metabolites: 4-aminobutyric acid, alanine, erythritol, fructose, glucose, glutamine, glycerol, lysine, ornithine and unknown-26.2880.

6.3.6 Metabolite composition of *S. nodorum* pycnidiospores.

To confirm links between metabolite abundance and sporulation observed in whole cultures, SN15 pycnidiospores were subjected to metabolite analysis by GC-MS. Pycnidiospores were harvested from SN15 grown on CZV8CS complete medium. Metabolites were extracted from the spores and analysed by GC-MS. Because of the small amount of tissue analysed, peak areas were calculated as a percentage of the total peak area, which normalises for changes in total metabolite amount. Four biological replicates were used. Fifty one different metabolites were identified, including amino acids, sugars, sugar alcohols, carboxylic acids and fatty acids (Figure 6.13 A). The amino acids included: glycine, alanine, leucine, isoleucine, valine, serine, threonine, phenylalanine, tyrosine, glutamic acid, glutamine and aspartic acid. One amino acid with a basic side chain was found, lysine. Proline, the imino acid was also identified. Other related amino acid metabolites were identified, beta-alanine, 4-hydroxyproline, pyroglutamic acid, 4-aminobutyric acid, and the putrescines, ornithine and spermidine.

Figure 6.13

The sugars found were: trehalose, maltose, fructose, myo-inositol, and glucose 6-phosphate. Sugar alcohols were highly abundant, including mannitol, xylitol/arabitol, glycerol and glycerol 3-phosphate. Carboxylic acids were found including citric acid, fumaric acid, malic acid and succinic acid. Fatty acids were well represented with hexadecanoic acid, octadecanoic acid, 9,12-octadecadienoic acid, 9,12,15-octadecatrienoic acid and eicosanoic acid all present. The remaining metabolites identified were: phosphate, urea and 2-aminoadipic acid, while nine other peaks could not be identified with certainty.

To reveal the major constituents of pycnidiospores, the metabolite areas (as a percent of the total area) were ranked from largest to smallest and plotted as a pie chart (Figure 6.13 B). This showed that together, mannitol (38%) and trehalose (22%) comprised well over half of the total peak area. The next four largest peak areas were: octadecanoic acid (6%), xylitol/arabitol (6%), hexadecanoic acid (5%), and glutamine (4%). The six largest peaks accounted for over 75% of the total area.

6.4 Discussion.

6.4.1 Method development - diluted samples.

A key problem encountered during the development of the GC-MS methodology was how to manage the massive dynamic range of the metabolome. The concentration of metabolites in biological systems has been estimated to cover up to 7-9 orders of magnitude (Dunn and Ellis, 2005). At 11 dpi and 18 dpi, the most abundant metabolite in SN15 accounted for almost 75% of the total peak area from the sample (Figure 6.5). This was a problem because it was important to record the area of the most abundant metabolites without saturating the column or the mass-spectrometer, and also to analyse enough sample to detect the low abundance metabolites, which included the majority of species. This problem was solved by running each sample twice, once using an undiluted sample to capture low abundance metabolites and a second time using a 50-fold diluted sample to correctly resolve high abundance metabolites. The ribitol internal standard corrected the dilution.

Analysis of undiluted and diluted samples showed that the retention time of a saturating mannitol peak could shift by 10 seconds or more (Figure 6.1). The area of the saturated peak was not representative of the amount of metabolite, in the case of mannitol, only 1% of the true abundance was described. The use of undiluted and 50-fold diluted samples enabled accurate quantitation of both high and low abundance metabolites.

6.4.2 Method development - RT locking

An enhancement to the initial protocol used in chapter 5 was the use of retention-time-locking chromatography. This process ensured that the retention of a defined

metabolite was reproducible, even after changing the column and altering its length. The RT-locking protocol greatly improved the accuracy of comparisons to libraries of authentic standards.

6.4.3 GC-MS analysis of *S. nodorum* SN15 grown *in vitro*.

SN15 was grown in minimal media and sampled at 4 dpi, 11 dpi and 18 dpi, representing three stages of growth and sporulation. Polar metabolites were extracted and analysed by GC-MS. 147 metabolites were detected over the three time points and 58 different metabolites were identified among those. The number of different metabolites detected was similar to the SN15-infected wheat study of chapter 5. A larger total number of metabolites were detected in this study, but a similar number were able to be identified. The number of identified metabolites was limited by the number of authentic standards available for comparison, use of larger libraries would undoubtedly have allowed identification of more unknown metabolites. The variation of metabolite abundances across biological replicates was generally much lower in the *in vitro* sporulation study when compared to the *in planta* sporulation study. This was probably because the amount of fungal and plant tissue could not be distinguished in the *in planta* study, nor could it be normalised as well. Infection of a living plant with a pathogen is a process that tends to progress in a non-linear fashion. The sudden collapse of a leaf can lead to the initial small differences between replicates becoming large differences once finished. HCA analysis of the metabolite abundances within each sample showed that GC-MS analysis was sufficient to correctly members of each time-point. This means that metabolomics was a good indicator of cellular changes that occurred during sporulation.

6.4.4 Distribution of metabolites across time-points

A comparison was performed whereby the metabolites identified in the *in planta* dataset were matched to those found in the *in vitro* dataset (Figure 6.14). Seventeen metabolites were only found in the *in planta* experiment, 22 were only found in the *in vitro* experiment and 35 were found in both. Unknown metabolites were not compared as the retention times were not equivalent. Well known plant metabolites (stigmasterol, beta-sitosterol and alpha-tocopherol) were only present in the *in planta* experiment while many low-abundance primary metabolites were only found in the *in vitro* data. These '*in vitro* only' metabolites included amino acids (arginine, cystathionine, homoglutamine, homoserine), sugars (raffinose, galactose and maltose), sugar phosphates (glucose 6-phosphate and fructose 6-phosphate) and sugar alcohols (erythritol, maltitol, and sorbitol). Possible reasons for the differences can be conclusively stated for examples like beta-sitosterol and stigmasterol which are plant sterols and are not found in fungi. The reason for the detection of sugar phosphates in only the *in vitro* grown SN15 samples is probably simply due to a higher concentration of metabolites analysed. *In vitro* fungal sample are 100 % fungal tissue, while *in planta* samples are mostly plant tissue, and contain a larger percentage of water. These factors may reduce the sugar phosphate conentration to below the limit of detection in the *in planta* samples. It may also be due to the growth media which contains sucrose as the sole carbon source. A large sucrose influx would result in a larger flow of carbon through glycolysis, in comparison to growth on plant tissue which is a far more complex growth substrate.

Figure 6.14

6.4.5 Sporulation associated metabolites

The aim of studying the metabolome of *S. nodorum* during sporulation *in vitro* was to find out which metabolites were positively associated with sporulation. Key metabolites grouped into two main groups, those that increased during sporulation and those that decreased. The metabolite abundances were analysed by two main methods, HCA and PCA. The HCA produced 10 clusters of metabolites based on their profiles, however they are not all homogenous groups. For example, mannitol is clustered with glycerol and glucose, two metabolites that decrease over time, while mannitol increases over time. The clustering was clearly biased by overall abundance rather than the change in concentration over time. The results of the PCA produced groups that were based more on the profile of abundance rather than the absolute amounts. PCA of the *in vitro* dataset showed that the first two components contained most of the variation within the samples, over 70% . The plot of factor loadings for each sample was able to resolve the three treatments, each component able to distinguish the time points. This meant that dissection of PC1 and PC2 factor loadings would provide a list of metabolites that characterised these biological situations. These two components were used to identify the metabolites that were positively correlated with sporulation, within groups 2, 3 and 4. (Figure 6.9). These three groups contained 76 metabolites, of which 30 were able to be identified. These metabolites were compared to the overall composition of SN15 pycnidiospores (Table 6.5, Figure 6.13), to determine the metabolites that were most positively correlated with sporulation.

The main metabolites associated with sporulation included trehalose, mannitol, octadecanoic acid, citric acid and malic acid. These were chosen on the basis of

abundance increasing as sporulation occurred, the statistical significance of those changes (Figure 6.12), and the presence of the metabolites in pycnidiospores.

Both trehalose and mannitol were identified as metabolites positively correlated with sporulation *in planta* in chapter 5, they were identified as being strongly correlated with sporulation *in vitro*, and have been shown to be highly abundant in spores themselves. This result confirmed their status as metabolites that are likely to be important for sporulation. As previously mentioned in chapter 5, mannitol has already been shown to be required for sporulation of *S. nodorum* both *in planta* and *in vitro* (Solomon *et al.*, 2006e). Trehalose has been shown to have a similar accumulation profile as mannitol, and in GC-MS analysis of pycnidiospores, represented over 20% of the total peak area. Trehalose is therefore the most interesting metabolite without prior study in *S. nodorum*. In addition, trehalose has been linked with sporulation in *M. grisea* (Foster *et al.*, 2003) and spore survival in *A. nidulans* (Fillinger *et al.*, 2001) making it an ideal candidate for further study. A more in-depth analysis of trehalose and its biological significance is given in chapter 7.

6.4.6 The metabolome of the *S. nodorum* pycnidiospore.

A preparation of pycnidiospores was analysed by GC-MS. The composition of pycnidiospores was generally more diverse than that of 11 dpi or 18 dpi entire cultures. The 4, 11 and 18 dpi cultures contained between three to five dominant metabolites that accounted for approximately 90% of the total peak area detected. In contrast, the pycnidiospore composition had more metabolites, each representing a smaller amount of the total metabolite pool. Some metabolites were more abundant in spores than in the 18 dpi entire culture samples including: trehalose (up 10-fold), octadecanoic acid (up

30-fold), hexadecanoic acid (up 50-fold), glutamine (up 130-fold), and 4-aminobutyric acid (up 65-fold).

Mannitol and trehalose were found in both *in planta* and *in vitro* experiments to be positively correlated with sporulation. The metabolite composition of the 18 dpi cultures, which were heavily sporulating, was 74% mannitol and 2% trehalose. In contrast, pycnidiospores contained 38 % mannitol and 22% trehalose. This suggests that trehalose is more specifically associated with spores, rather than general hyphal growth of the colony.

6.4.7 Lipid metabolism and the asexual life cycle of *S. nodorum*.

The abundance of each metabolite was projected onto a basic metabolic map of primary metabolism, including some relevant side pathways. This provided a general overview of how different metabolites were enzymatically linked, and added a layer of biological significance to the data.

An overall appraisal of metabolic changes during sporulation revealed lipid biosynthesis was one of the dominant features of the primary metabolism map (Figure 6.15A, B). The metabolites with interesting abundance profiles included the fatty acids octadecanoic acid (syn. stearic acid) and 9,12-octadecadienoic acid (linoleic acid) and 9,12,15-octadecanoic acid (linolenic acid). They all increased in the *in vitro* cultures as sporulation occurred, with statistically validated changes in mean abundance (Figure Table 6.3, 6.4), additionally, all but 9,12,15-octadecatrienoic acid were present in large amounts in pycnidiospores (Figure 6.13). Another lipid-related metabolite, 3-phosphoglycerate, was shown to increase at 11 and 18 dpi. Almost all of the members TCA cycle, minus oxaloacetate and succinyl CoA which were not detected, generally

Figure 6.15 a

Figure 6.15 b

increased in abundance at 11 dpi and 18 dpi. This is consistent with an influx of acetate into the TCA cycle via acetyl-CoA from fatty acid beta-oxidation. By 11 dpi, citrate was the most abundant member of the TCA cycle at around $3x10^7$ abundance units, malate was the next most abundant at $9x10^6$ units, and all the other members were one or two orders of magnitude lower than citrate.

A high concentration of citrate stimulates fatty acid biosynthesis via allosteric activation of acetyl-CoA carboxylase (Stryer, 1996). This enzyme catalyses the formation of malonyl-CoA from acetyl-CoA, the first committed step in fatty acid biosynthesis. The abundance of citrate matched the hypothesis that cellular metabolism had shifted to lipid biosynthesis by 11 dpi, when sporulation was occurring in samples. A metabolite with a partial MST match to malonate was found at 11.1103 min. The match was very close, scored as 95 out of 100. This potential malonate metabolite also increased at 11 and 18 dpi, in concert with citrate and fatty acids. It was probably formed from malonyl-CoA as an artefactual degradation product caused by sample preparation. However, despite the lack of an obvious enzymatic step to produce free malonate, it is still an indication of the amount of malonate or malonyl-CoA present at the time.

6.4.8 Glycerol biosynthesis and early growth of *S. nodorum*.

Glycerol is required for the biosynthesis of triacylglycerides (TAGs), and is released when they are catabolized. Glycerol was observed to be highly abundant at 4 dpi, before sporulation was occurring, and had almost disappeared at 11 dpi and 18 dpi. At the same time, as glycerol concentrations dropped, primary metabolism shifted to produce more lipid, presumably to supply the nascent spores. These patterns of abundance were in accord with a study that showed, in *S. nodorum*, malate synthase activity and hence lipid breakdown and gluconeogenesis, was essential for germination

of spores in the absence of an external carbon source (Solomon *et al.*, 2004a). The large amount of glycerol present at 4 dpi may be the result of active biosynthesis of glycerol from the products of beta-oxidation of fatty acids via gluconeogenesis, as well as from release of glycerol from lipolysis of TAGs.

It is interesting to consider that while two necrotrophs, *Magnaporthe grisea* and *S. nodorum,* both produce glycerol during the early stage of growth, they do not appear to put it to the same use. *M. grisea* has been suggested to use the abundant glycogen trehalose, lipid stores in conidia to produce high concentrations of glycerol (Thines *et al.*, 2000). Of these three storage metabolites, glycogen is thought to be the main energy source in conidia in *M . grisea* (Thines *et al.*, 2000). This theory was supported by the observation that deletion of the gene encoding isocitrate lyase only delayed germination and appressoria formation. It appears that lipid degradation is used for glycerol biosynthesis but is not essential for it to occur in *M .grisea* (Wang *et al.*, 2003). As mentioned earlier, the storage metabolites are used to produce glycerol, which is sequestered within an appressorium and generates the turgor pressure required to drive a penetration peg through the host plant cuticle.

S. nodorum has been shown to penetrate its wheat host without the use of an appressorium (Solomon *et al.*, 2006f), yet it still produces large amounts of glycerol after germination and quickly removes it soon afterwards. The purpose of such abundant glycerol is not yet known in *S. nodorum*. It may be produced simply as a by-product of lipid breakdown. Perhaps lipolysis and glycerol accumulation during spore germination is an ancient process, and as *M. grisea* evolved and diverged into its own

biological niche, it adapted a by-product of lipid degradation (glycerol) to a more specialised use.

In a similar fashion to *S. nodorum*, *A. nidulans* accumulates glycerol to high concentrations soon after germination (de Vries *et al.*, 2003). Loss of the *GldB* gene, encoding a glycerol dehydrogenase, resulted in mutant strains that could not produce glycerol and were sensitive to osmotic stress. It may be that glycerol accumulates simply as a result of rapid lipid degradation, but perhaps *S. nodorum* harnesses the released glycerol for osmotic protection. It may be that glycerol is the most efficient way for hyphae to generate osmotic potential during early growth. Osmotic potential is largely driven by solute concentration, which means that a smaller solute (glycerol) can produce the same potential as a larger solute (mannitol) for less carbon input (Davis *et al.*, 2000). The loss of glycerol from the *S. nodorum* cell coincided with the production of two larger polyols, mannitol and xylitol/arabitol, which accumulated to even higher levels than glycerol did. These "late-polyols" may have provided better general protection, perhaps including other areas of cell protection, such as oxidative stress. Conversely, it may be that certain metabolites must be absent for sporulation to occur normally. It would be interesting to see if the artificial upregulation of glycerol biosynthesis in *S. nodorum* was sufficient to prevent sporulation of the fungus.

The fatty acids detected during *in vitro* growth and in pycnidiospores alone, could be breakdown products of *S. nodorum* triacyl glycerides (TAGs), or they may be precursor molecules for larger more complex lipids. They could also arise from artificial degradation of phospholipids or TAGs during sample preparation. Although the precise

role for these fatty acids cannot be stated, they can still be considered under the umbrella of general lipid biosynthesis and breakdown.

Lipid has been identified as an essential energy reserve of *S. nodorum* pycnidiospores (Solomon *et al.*, 2004a). In yeast these lipids are stored in lipid bodies and are predominantly in the form of triacylglycerols and stearyl esters (Zweytick *et al.*, 2000). Fatty acids identified in lipid bodies of *Yarrowia lipolytica* included hexadecanoic acid, octadecanoic acid, and predominantly 9-octadecanoic acid and 9,12- octadecanoic acid (Athenstaedt *et al.*, 2006). Interestingly, the two saturated fatty acids (hexadecanoic and octadecanoic acid) had very similar abundance profiles over the three timepoints, as did the unsaturated fatty acids, with a different profile. This suggests there may be divergent regulation for the two types of lipid. Perhaps one form predominates as a storage lipid, while the other is more common as a membrane lipid.

As spores have a large surface area to volume ratio when compared to hyphae, membrane lipid biosynthesis should increase as sporulation occurs *en masse*. It may be that in order to produce a large number of spores, (such as those found within a pycnidium), the lipid biosynthetic pathways must upregulate, in order to supply the increased demand for cell membrane components in addition to lipid bodies. Ergosterol is a well known component of fungal cell walls (Seitz *et al.*, 1979), yet it was not observed in these studies, even as a partial mass-spectral match. The absence of ergosterol was probably due to the unsuitable nature of the analytical system for lipid analysis. GC-MS is not suited to analysis of ergosterol, as analysis of an authentic standard showed that the response was very inefficient (data not shown), suggesting

cellular levels of ergosterol would not be detected, even if it had been efficiently extracted from tissue.

Yet another role for fatty acids has been described in *A. nidulans*. Psi-factor unsaturated fatty-acids can control the ratio of asexual to sexual spores (Calvo *et al.*, 2001; Tsitsigiannis and Keller, 2006). These psi-factors are derived from oleic (9-octadecenoic acid) and linoleic acid (9,12-octadecadienoic acid). Three fatty-acid oxygenase enzymes are responsible for the required biosynthetic steps, PpoA, PpoB, and PpoC. Deletion of all three *Ppo* genes in *A. nidulans* resulted in increased, and miss-scheduled activation of sexual sporulation. Interestingly, BlastP alignment of the three *A. nidulans* Ppo amino acid sequences with the auto-annotated protein set of *S. nodorum* showed that PpoA was the most related of the three with *S. nodorum* sequences (Table 6.6). There were two closely related sequences in *S. nodorum*, but there was not a third with significant homology. The high degree of similarity suggests a high likelihood of a conserved role in *S. nodorum.* There have not been any studies in *S. nodorum* on the role of fatty acid oxygenases on sporulation.

Table 6.6

BlastP alignment of *A. nidulans* PpoA, PpoB and PpoC to the *S. nodorum* protein set.

	Degree of alignment to *S. nodorum* SN15 sequences.		
	PpoA	**PpoB**	**PpoC**
SNOG_07393	50%, 1097 bits	36%, 589 bits	44%, 890 bits
SNOG_03918	34%, 406 bits	26%, 288 bits	30%, 422 bits

The *A. nidulans* protein sequences PpoA (AAR88626), PpoB (AAX35769) and PpoC (AAT36614) were compared to the *S. nodorum* protein set using the BlastP algorithm. The top two matches for each search are summarised in this table. % refers to the degree of percent identity between the two amino acid sequences. Bits refer to the bit score of the alignment between the two sequences.

6.4.9 Summary

It has been shown that the *S. nodorum* metabolome was able to be thoroughly investigated using GC-MS. Over 150 metabolites were separated and quantified, and approximately 50 were formally identified. Lipid metabolism was revealed as a overriding theme in metabolic changes observed during sporulation. Observations matched current theories from the degradation of lipid after germination, to the re-synthesis of lipid to re-supply the asexual spores. Within the 150 detected metabolites, both mannitol and trehalose stood out as key metabolites during the process of asexual development, both *in vitro* and *in planta*. Their accumulation was rapid and positively correlated with the onset of sporulation. This link to sporulation was supported by analysis of pycnidiospores, which showed both mannitol and trehalose were major constituents. As mannitol has already been shown to be required for asexual sporulation of *S. nodorum*, trehalose was chosen as a candidate metabolite for further study. Its role during sporulation was investigated by a reverse genetics approach.

Chapter 7

Trehalose biosynthesis and sporulation in *Stagonospora nodorum*

7.1 Introduction

7.1.1 Trehalose structure.

Trehalose is a disaccharide found in a diverse range of life forms. The trehalose molecule consists of two glucose monomers joined by an alpha 1-1 linkage, creating a non-reducing sugar (Figure 7.1). Trehalose has been identified in mammals, plants, insects and fungi (Elbein *et al.*, 2003).

There is a long history of research involving trehalose. It was originally isolated from two distinct sources during the 1800's. Wiggers published a description of the constituents of ergot of rye (*Claviceps pupurea*) in 1832, which included a non-reducing sugar that would be later identified as mycose (Wiggers, 1832). The name mycose was later discarded in favour of trehalose (Elbein *et al.*, 2003). The name trehalose was first used in 1858 to describe a non-reducing sugar isolated from "trehala manna", the discarded cocoons of the old world beetle *Larinus maculatus* (O'Neil, 2001). The shells are composed of approximately 25-30 % trehalose.

7.1.2 Trehalose biosynthesis and degradation.

Trehalose is biosynthesised by several different pathways in different phyla (Figure 7.2). Since this study concerns an ascomycete fungus, it will be discussed in this context first.

Figure 7.1

Figure 7.2

7.1.3 The OtsA/B pathway.

Trehalose biosynthesis was first described in *Saccharomyces cerevisiae* (Cabib and Leloir, 1958; Leloir and Cabib, 1953), and is as follows. The first step is condensation of glucose 6-phosphate and UDP-glucose to trehalose 6-phosphate, catalysed by trehalose 6-phosphate synthase (Tps1) (EC 2.4.1.15). Then, trehalose 6-phosphate is dephosphorylated by trehalose 6-phosphate phosphatase (Tpp1) (EC 3.1.3.12) to produce trehalose and inorganic phosphate (P_i). The pathway in *E. coli* is catalysed by OtsA, a Tps1, and OtsB, a Tpp1. The synthesis of trehalose via trehalose 6-phosphate is commonly referred to as the OtsA/B pathway.

In fungi, trehalose can be broken down into two molecules of glucose by the action of trehalase (EC 3.2.1.28) which is usually present in two forms, cytosolic or neutral trehalase and vacuolar or acid trehalase (Elbein *et al.*, 2003). Trehalose can be rapidly degraded after accumulation to high concentrations. For example, in *M. grisea*, trehalose breakdown in germinating conidia was monitored and showed that the wild-type Guy11 strain almost completely degraded its conidial trehalose stores within two hours of germination, trehalose levels in mutants lacking the Tre1 trehalase did not reduce over the same period (Foster *et al.*, 2003). The same rapid degradation is also seen in yeast after heat stressed cultures are returned to normal growth conditions (Singer and Lindquist, 1998b).

In *Saccharomyces cerevisiae,* the enzymes required for trehalose biosynthesis are associated in a multi-protein complex consisting of trehalose 6-phosphate synthase (TPS1), trehalose 6-phosphate phosphorylase (TPS2) and two regulatory subunits TPS3, and TSL1 (Bell *et al.*, 1998). All subunits are required for full activity, with TSL1 and

TPS3 shown to be required for stability of the complex. This is in contrast to the related enzymes in *E. coli*, where OtsA (TPS1 homologue) and OtsB (TPS2 homologue) are not found as a complex (Giaever *et al.*, 1988; Kaasen *et al.*, 1994).

Interestingly, the members of the yeast trehalose complex all share sequence homology, with Tps1 sharing approximately 35% identity with the three other members of the complex (Elbein *et al.*, 2003).

7.1.3.1 The TreY/Z and TreS pathways.

Two other pathways to synthesise trehalose have been elucidated. The TreY/Z pathway begins with the rearrangement of glucose polymer (for example, glycogen) by TreY a maltooligosyl trehalose synthase to produce maltooligosyl trehalose (MOT). TreZ, a MOT hydrolase, then releases trehalose from the glucose polymer. A third pathway involves a single enzyme, TreS or trehalose synthase. TreS catalyses the rearrangement of maltose to trehalose. Interestingly, *Mycobacterium tuberculosis* contains both TreY/Z, TreS and OtsA/B pathways (De Smet *et al.*, 2000). In addition to the TreY/Z and TreS activities, two different trehalose phosphorylase activities have been described in the fungi *Schizophyllum commune* (EC 2.4.1.231) and the protist *Euglena gracilis* (EC 2.4.1.64) (Belocopitow and Marechal, 1970; Nidetzky and Eis, 2001). These enzymes can convert trehalose and inorganic phosphate to glucose and glucose 1-phosphate (Figure 7.2).

7.1.4 Roles of trehalose with the cell.

The role of trehalose in biology is complex with multiple roles, often associated with stress protection, metabolic regulation and energy storage. Trehalose has also been implicated in the pathogenicity of plant and animal pathogens.

7.1.4.1 Trehalose biosynthesis as a control point for glycolysis.

Tps1 has found to be necessary for growth of *S. cerevisiae* on fructose or glucose as the sole carbon source. Mutants lacking *Tps1* had elevated levels of sugar phosphates and depleted ATP and Pi concentrations, which is thought to explain the inability to grow on these carbon sources. Uncontrolled entry of glycolytic substrates appears to result in rapid depletion of ATP. Trehalose 6-phosphate has been shown to strongly inhibit the most active hexokinase in yeast. It was postulated that the formation of trehalose 6-phosphate helped to prevent uncontrolled entry of glucose or fructose into glycolysis, and the exhaustion of ATP and P_i (Blazquez *et al.*, 1993). Interestingly, the *tps1* growth defect could be repaired by a mutation in the cytochrome b5 complex, resulting in the production of excess glycerol. In this case, the production of glycerol was thought to provide sufficient NAD+ and P_i for glyceraldehyde-3-phosphate dehydrogenase activity (Blazquez and Gancedo, 1995).

7.1.4.2 Desiccation stress.

Trehalose has been suggested to provide protection against desiccation damage. Yeast cultures with elevated trehalose levels have been correlated with greater rates of survival after desiccation. Exogenously applied trehalose provided a similar protective effect (Gadd *et al.*, 1987). However, a recent study has revealed that *S. cerevisiae tps1* mutants, which completely lacked trehalose, still retained some desiccation tolerance. And conversely, wild-type cultures which were pre-conditioned with an osmotic shock, contained greater trehalose concentrations than non-conditioned cultures, yet had lower desiccation survival rates (Ratnakumar and Tunnacliffe, 2006). It is clear that the subject has not yet been resolved, although it appears that use of yeast as a model for trehalose-mediated stress response may be fraught with problems caused by the requirement of Tps1 activity for proper glycolytic function.

7.1.4.3 Heat stress.

Trehalose has been shown to be correlated with a significant heat stress protective effect in yeast (De Virgilio *et al.*, 1994; Hottiger *et al.*, 1994). *Tps1* mutants could not accumulate trehalose upon heat shock, and had less resistance to heat stress than a wild-type strain. As the effect was postulated to occur via protection of proteins, sugars, polyols and amino acids were tested for their ability to stabilise proteins at high temperatures, trehalose was shown to be as good or better than all the solutes tested.

In vivo evidence suggests trehalose may play a minor role in heat stress response. Yeast *tps1* mutants have been discovered to have reduced levels of protective heat-shock proteins (HSPs), which prompted the authors to suggest that the loss of heat shock tolerance was solely due to loss of HSPs (Hazell *et al.*, 1995). It may be that trehalose provides a short term protective effect, long enough to allow HSP production and full protection (Singer and Lindquist, 1998b). A related study showed that trehalose interacted with native yeast proteins to help maintain their tertiary structure and, unexpectedly, also bound to denatured proteins and prevented their aggregation (Singer and Lindquist, 1998a). Interestingly, the trehalose-protected proteins had to be stripped of trehalose before they could be refolded by the HSP chaperone system .

The protective effect of high concentrations of trehalose has also been suggested to be due to an increase in cellular viscosity, which reduces molecular motion and hence protein denaturation (Sampedro and Uribe, 2004). The increase in viscosity also correlated with a decrease in the *in vitro* enzyme activity of an H(+)-ATPase from *Kluyveromyces lactis*. These observations both fit with the rapid production of trehalose by *S. cerevisiae* upon heat shock, and subsequent rapid degradation of

trehalose once the heat shock has been removed (Elbein *et al.*, 2003). Although, in the plant pathogen *M. grisea*, deletion of *Tps1* did not effect its survival after heat shock (Foster *et al.*, 2003).

7.1.4.4 Oxidative stress.

Trehalose has been reported to be involved in cellular defence against oxidative stress. In *Candida albicans*, loss of *Tps1* resulted in mutant strains that were more sensitive to hydrogen peroxide treatment (Gonzalez-Parraga *et al.*, 2003). The increased sensitivity was concomitant with a greater induction of known antioxidant stress enzymes, such as catalase and super oxide dismutase. It was suggested the induction of defence enzymes was an attempt to compensate for the loss of trehalose biosynthesis.

In *Aspergillus nidulans*, loss of *Tps1* and trehalose biosynthesis, was observed to result in increased sensitivity of germlings to moderate oxidative stress (2 mM H_2O_2), but had no effect on resistance to severe oxidative stress (100 mM H_2O_2) (Fillinger *et al.*, 2001).

7.1.4.5 Osmotic stress.

Stress caused by high concentrations of external osmolytes can be relieved by trehalose. Trehalose concentrations were reported to correlate with tolerance to salt stress in the photosynthetic bacterium *Rhodobacter sphaeroides* (Makihara *et al.*, 2005). In addition to microbes, trehalose has been shown to alleviate stress in plants. Garg *et al.* (2002) showed that overproduction of trehalose in rice plants conferred greater tolerance to salt stress and drought stress. Although trehalose accumulated in the transgenic plants to 3-10 time the concentration of wild-type plants, it was not considered high enough for the effect to be caused by trehalose acting as a compatible solute. Rather, the effect was

thought to be due to modified carbohydrate metabolism and elevated photosynthetic rates.

7.1.4.6 Carbohydrate store.

The presence of trehalose in high concentrations in many fungi has lead to the suggestion it functions as storage carbohydrate (Thevelein, 1984). In *Neurospora* ascospores, it has been shown that lipids are consumed during dormancy and trehalose is consumed upon germination (Lingappa and Sussman, 1959). The consumption of trehalose upon germination is also seen in the plant-pathogens *M. grisea* and *B. cinerea* (Doehlemann *et al.*, 2006; Foster *et al.*, 2003). Although trehalose does provide energy when catabolized, in yeast, glycogen is catabolised first, and then trehalose is degraded at the onset of cell death (Singer and Lindquist, 1998b). This suggests trehalose is not a preferred storage carbohydrate in yeast

7.1.4.7 Pathogenicity.

A great deal of research has been conducted on the role trehalose plays in free living organisms such as yeasts and saprophytes such as *Neurospora crassa* and *Aspergillus nidulans,* yet comparatively little is known of the role it plays in pathogenic fungi. *Candida albicans* is an opportunistic pathogen of humans (Mitchell, 1998). It has been shown that *C. albicans* requires trehalose biosynthesis for infectivity and virulence on mice (Zaragoza *et al.*, 1998). Strains lacking *Tps1* did not undergo transformation from yeast-like growth to the virulent hyphal-growth stage when grown at 37ºC.

Both the biosynthesis and degradation of trehalose has been shown to be important during the life-cycle of *M. grisea*, a rice pathogen (Foster *et al.*, 2003). Mutation of *Tps1* and loss of trehalose biosynthesis prevented the formation of appressoria and

penetration of the leaf cuticle. If the cuticle was removed, infection was able to proceed in mutants. Trehalose breakdown via Nth1 was not required for penetration, but was required for rapid colonisation of the host tissue. Tre1, a second trehalase, was found to be dispensable for pathogenicity on rice. Pathogenicity of the fungal plant pathogen, *Botrytis cinerea* was not affected by the loss of trehalose biosynthesis (*tps1*) or degradation (*tre1*) (Doehlemann *et al.*, 2006). The *tps1* mutants were more sensitive to heat stress and showed germination defects.

Trehalose biosynthesis and virulence has been studied in *Cryptococcus neoformans.* It was shown that trehalose biosynthesis was required for virulence on both mice and *Caenorhabditis elegans* (Petzold *et al.*, 2006).

7.1.4.8 Sporulation.

As this study primarily concerns sporulation of a plant pathogen, the role of trehalose in sporulation is of interest. Only one study of a plant fungal pathogen has shown that trehalose biosynthesis is required for sporulation. Forster *et. al.* (Foster *et al.*, 2003) showed that deletion of *Tps1* in *M. grisea* resulted in a large reduction in sporulation in culture, from 1.39×10^6 conidia per plate for wild-type down to 3.6×10^3 for the *tps1* strain. Outside of the plant pathogens, studies showing a trehalose dependant effect on sporulation are rare. Yeast strains lacking *Tps1* fail to produce meiotic inducers, IME1, IME2 and MCK1, and sporulate poorly (De Silva-Udawatta and Cannon, 2001). It was suggested that *Tps1* was required for full induction of sporulation.

Overall, it appears that a definitive role for trehalose in pathogenicity does not exist. In different organisms, loss of trehalose metabolism may cause loss of pathogenicity,

sporulation or stress response, but no consistent picture across all examples has emerged.

7.1.5 This study.

Previously, the abundance of trehalose was shown to be closely associated with sporulation of *Stagonospora nodorum* and was identified as the major metabolite of interest from *in planta* and *in vitro* metabolomics studies (Chapters 5 and 6). As the role of trehalose biosynthesis in sporulation or pathogenicity had not yet been determined in this pathogen, it was selected for further study by targeted gene replacement.

7.2 Materials and methods.

7.2.1 Liquid culture growth assay.

Minimal medium (200 μL) was inoculated with 5000 pycnidiospores. Eight replicates were performed per strain, using a 96-well microtitre plate format. Plates were sealed and incubated at 20ºC until 7 dpi. OD 595 nm was recorded before and after the incubation steps and the net change in OD595 was calculated. Net OD595 was used to infer growth rate of the strain as used by Solomon *et al.* (2003). Further media additions were noted in each case.

7.2.2 Oxidative stress assay.

Strains were grown as for a liquid culture growth assay, but with media additions of 0 mM, 0.03 mM or 0.1 mM t-butyl-peroxide (Sigma) as an oxidant.

7.2.3 Heat stress assay.

Strains were grown as for a liquid culture growth assay, but with incubations performed at 20ºC, 30 ºC, 35 ºC and 37ºC.

7.2.4 Germination assay.

7.2.4.1 Thin agarose slides.

Two cover slips were placed on either end of a glass microscope slide, and ~50 μL of molten agarose (1% w/v) was placed in the centre of the slide. A long cover slip was then quickly placed over the drop of agarose, spreading it thinly. The agarose was allowed to set and the coverslips were carefully removed, leaving a thin, uniform, agarose layer deposited on the slide.

7.2.4.2 Germination procedure.

Pycnidiospores (approximately 5000) were deposited onto a thin agarose slide and incubated in a humid chamber at the desired temperature. Incubations were performed overnight to ensure full germination occurred. Spores with a visible germ tube were considered germinated.

7.2.5 SIM mode GC-MS.

SIM mode GC-MS was performed similarly to GC-MS previously described, with the following changes. Mass spectral ion acquisition was changed from SCAN mode to SIM mode. Instead of collecting entire spectra, three ions were monitored, m/z 319 (base peak trehalose-TMS), m/z 217 (base peak mannitol-TMS) and m/z 191 (base peak ribitol-TMS). The dwell time for each ion was set to 20 ms. The settings allowed an increase in sensitivity, approximately 20-fold greater than that achieved in full scan mode. SIM mode is appropriate for quantitation of metabolites in limiting amounts of sample.

7.2.6 Bioinformatics.

7.2.6.1 Sequence similarity searches.

Blast comparisons were performed at the NCBI website www.ncbi.nlm.nih.gov and the Broad institute website www.broad.mit.edu. BlastX and BlastP algorithms were used for comparisons (Altschul *et al.*, 1997). COG and PFAM domains were identified using the Conserved Domain Database and Search Service, v2.09 at the NCBI website (Marchler-Bauer *et al.*, 2005).

7.2.6.2 Multiple sequence alignment.

Multiple sequence alignments were performed using the EBI ClustalW service, available at the website www.ebi.co.uk (Myers and Miller, 1988; Wilbur and Lipman, 1983). Alignments were performed using the default settings. Amino acid sequence alignments were shaded according to the ClustalX aminoacid properties.

7.2.6.3 Phylogenetic tree building.

Phylogenetic trees were calculated from ClustalW multiple sequence alignments. Alignments were processed by Jalview software using default parameters (Clamp *et al.*, 2004). Trees were calculated as average distance trees using the Blosum62 algorithm, distances are shown.

7.2.6.4 *S. nodorum* SN15 genome sequence.

The *S. nodorum* SN15 genome sequence was accessed at the Broad institute website www.broad.mit.edu, and at the NCBI website www.ncbi.nlm.nih.gov. Raw sequence data was retrieved from the NCBI trace archive at the NCBI website. Chromatograms were individually viewed using FinchTV sequence viewer (www.geospiza.com).

7.2.6.5 Contig building.

Sequence contigs were built using Vector NTI 10 / Contig express software (Invitrogen).

7.3 Results.

7.3.1 Identification of the gene encoding trehalose 6-phosphate synthase.

The first committed step in trehalose biosynthesis is the formation of trehalose 6-phosphate from UDP-glucose and glucose 6-phosphate by trehalose 6-phosphate synthase (TPS). This enzymatic step is a key metabolic control point, which makes the gene encoding trehalose 6-phosphate synthase an ideal candidate for mutagenesis. The *S. nodorum* genome sequence was searched for genes likely to encode such an enzyme.

To find all of the *S. nodorum* genes that could potentially encode a TPS enzyme, the TBlastN algorithm was used to compare the *S. cerevisiae* Tps1 protein sequence (AAT27376) to the S. *nodorum* SN15 genome sequence. The *S. cerevisiae* Tps1 was used because it is the most studied fungal Tps enzyme. It aligned to three regions of similarity, found on bases 364242-364985 bp of scaffold 15 (Genbank accession CH445339), bases 102204-103538 bp of scaffold 5 (Genbank accession CH445329) and bases 418699-420024 bp of scaffold 4 (Genbank accession CH445328). Each of these loci corresponded to an auto-called gene, SNOG_09603, SNOG_03369, SNOG_02747, respectively. BlastP comparison of the *S. cerevisiae* Tps1 protein sequence to the *S. nodorum* predicted protein set confirmed these loci were the only candidates for a *S. nodorum Tps1* homologue, based on sequence similarity. The BlastP results showed the following degrees of alignment with *S. cerevisiae* Tps1; SNOG_09603 63% identity, SNOG_03369 37% identity, SNOG_02747 38% identity.

BlastP comparison of the best Tps1 hit, SNOG_09603, protein sequence with the Swissprot database revealed many strong alignments with other fungal sequences

encoding experimentally validated alpha-alpha-trehalose 6-phosphate synthases. The results also showed there was strong conservation at the amino acid level among fungal TPS enzymes. The top hit to SNOG_09603 was alpha,alpha-trehalose-phosphate synthase (Acc: O59921) from *Emericella nidulans* that shared 72% identity with SNOG_09603 at the amino acid level.

In order to identify the conserved domains in SNOG_09603 pfam and COG groups were searched at the Conserved Domain Search service (v 2.08) (Marchler-Bauer *et al.*, 2002; Marchler-Bauer *et al.*, 2005) at the NCBI website. It showed SNOG_09360 had 72% alignment with the pfam domain Glyco_transf_20 (pfam00982), which is always associated with TPS enzymes (Figure 7.3 A). In addition, the COG group OtsA Trehalose-6-phosphate synthase (COG0380) aligned 74% with SNOG_09603. Therefore, the gene SNOG_09603 was named *Tps1*. The analysis revealed the auto-annotated amino acid sequence for *Tps1* contained a truncation of the OtsA pfam domain at the N-terminus.

It is unlikely that a gene would contain a truncated pfam domain, considering the high degree of conservation within the rest of the sequence. The sequence chromatograms for the *Tps1* coding region were examined to confirm the nucleotide sequence and the translation. It was found that there was a region of low sequence quality near the break in amino acid sequence homology. There were only a few sequence traces covering this region and the automated consensus builder had incorrectly interpreted the chromatograms. Two sequencing errors were identified. In the first error, the auto-annotated sequence ACG GCT:*GGG*:CCG GGT was corrected to ACG GCT:*GG*:CCG GGT (Figure 7.4 A).

Figure 7.3

Figure 7.4

In the second error, the auto-annotated sequence GCG GAC:*CAGA*:CAC TAC was corrected to GCG GAC:*CTG*:CAC TAC (Figure 7.4 B). After correction of sequencing errors, the nucleotide sequence encoded a full length OtsA pfam domain (Figure 7.3 B).

The manually annotated Tps1 protein sequence had a better BlastP alignment to the *Emericella nidulans* Tps1 (O59921) sequence (79%) compared to the auto-annotation (72%). Based on the BlastP sequence alignment to characterised Tps1 sequences, the revised annotation for Tps1 was adopted as the definitive version of SNOG_09603.

An average distance phylogenetic tree was constructed from the three *S. nodorum* sequences that best aligned to *S. cerevisiae* Tps1, plus other trehalose biosynthetic and degradation enzymes (Figure 7.5). Other sequences were selected from closely related fungi, where experimental evidence for function was available in almost all cases. An additional two *S. nodorum* proteins were included, representing proteins related to trehalases. BlastP alignment of *M. grisea* Tre1 (accession AAN38003) and *M. grisea* Nth1 (accession AAN46743) was used to select possible trehalase sequences from the *S. nodorum* genome sequence. SNOG_01591 was identified as the most similar protein to Nth1 of *M. grisea,* a confirmed neutral trehalase. SNOG_12745 was identified as the protein most similar to *M. grisea* Tre1, a novel trehalase which doesn't group with either neutral or acidic trehalases. A statistically significant match to *E. nidulans* Ath1, an acid trehalase, was not found in the *S. nodorum* genome.

The phylogenetic tree showed that SNOG_09603 grouped with other confirmed Tps1 sequences. The other two potential Tps1 sequences, SNOG_02747 and SNOG_03369, grouped apart, instead grouping with trehalose 6-phosphate phosphorylases and Tps3

Figure 7.5

regulatory proteins, respectively. In addition, the tree suggested that SNOG_12745 and SNOG-01591 were possible candidates for Tre1 and neutral trehalases, respectively.

To determine if *S. nodorum* Tps1 had any significant amino acid changes relative to other characterised Tps1 enzymes, a multiple sequence alignment was performed using Tps1 amino acid sequences from related fungi (Figure 7.6). The *Escherichia coli* OtsA sequence was included as it was the only trehalose 6-phosphate synthase for which a tertiary structure was available. The alignment showed that *S. nodorum* Tps1 had all of the highly-conserved amino acids required for trehalose 6-phosphate synthase activity. In particular, the key residues for Wrabl and Grishin's "glycogen phosphorylase glycosyltransferase" motif were present (Wrabl and Grishin, 2001). The tertiary structure of *E. coli* OtsA was determined by Gibson *et. al*. (2002), who identified the key residues required for trehalose 6-phosphate synthase activity. The tertiary structure of OtsA in complex with substrates, identified conserved residues involved in substrate binding; glucose 6-phosphate was bound by residues Arg9, Trp40, Tyr76, Trp85 and Arg300 (highlighted by a red box) while UDP-glucose was bound by residues Gly22, Asp130, His154, Arg262, Asp361 and Glu369 (highlighted by a green box). These key residues were all found in the predicted sequence for *S. nodorum* Tps1.

7.3.2 Disruption of the gene encoding trehalose 6-phosphate synthase.

To determine whether Tps1 is required for trehalose biosynthesis in *S. nodorum*, the gene was removed from the wild-type strain SN15 using targeted gene replacement.

Figure 7.6 a

Figure 7.6

A 1056 bp region 5' of the *Tps1* start codon was amplified using primers TPSKO5'F (5'-CTCGAGAGATCTAATAGATGCCATAA-3') and TPSKO5'R (5'-AAGCTTTGTCATGTTTGCGGTATATA-3') and a 1119 bp region 3' of the *Tps1* stop codon was amplified using primers TPSKO3'F (5'-GCAGAGCAGCTCCCTTGCCGTTCT-3') and TPSKO3'R (5'-GCGGCCGCTCTATAGATGGTGTACAGTC-3'). The PCR product from each flanking region was initially cloned into pGEM-t-easy, creating the pGEM-TPS-5' and pGEM-TPS-3' clones. The pGEM-Tps-3' clone was digested with *Pst*I and *Not*I and the released 3' flank cloned into *Pst*I and *Not*I sites of pBSK-phleo, creating the plasmid pBSK-phleo-Tps-3'. The pGEM-Tps-5' clone was then digested with *Xho*I and *Hin*dIII and the released *Tps1* 5' flank cloned into *Xho*I and *Hin*dIII sites of pBSK-phleo-Tps-3', creating pTPSKO (Figure 7.7).

The knockout vector was sequenced using primers TPSKO5'F, TPSKO5'R, M13F and M13 R, TPSKO3'F and TPSKO3'R. These primers were predicted to produce sequence covering the ligation sites of the construct. The M13F sequencing reaction failed, all others were successful. The sequence chromatograms showed that ligation of the 5' flank into *Xho*I and *Hin*dIII restriction sites had occurred as predicted (data not shown). The ligation of the 3' TPS flank was successful for the NotI site, but the *Pst*I site at the 5' end of the 3' flank had been unexpectedly modified (data not shown). The *Pst*I site was destroyed, as a four base-pair deletion has occurred within the six bp site. The predicted sequence TTC CTGCAG AGC was present as TTC CG AGC in the construct. No other unexpected modifications were found within the construct.

Figure 7.7

The knockout vector, pTPSKO, was linearised with *Xho*I and used to transform SN15 protoplasts. Genomic DNA isolated from 65 phleomycin resistant colonies was screened by PCR amplification of the entire *Tps1* locus (Figure 7.8). The presence of a larger 2.6 kb amplicon indicated the phleomycin cassette had replaced the coding sequence for *Tps1*. Positive clones were further analysed by Southern blot to check the nature of the integration event (Figure 7.9). The hybridisation probe was a 150 bp region of the pTPSKO 3' flank amplified using primers TPSKO3'F and TPS-Probe-3'. The PCR product was used as template for a random-primed digoxygenin-11-dUTP random labelled hybridisation probe. Genomic DNA (5 µg) from a selection of phleomycin resistant isolates was analysed. The probe was predicted to hybridise to a 0.9 kb fragment in wild-type strains; to a 1.5 Kb fragment in strains containing a targeted replacement of *Tps1* with pTPSKO; and to both a 0.9 kb and a 1.5 Kb fragment in strains containing a non-targeted integration of pTPSKO. Strains *tps1*-9, *tps1*-13, *tps1*-15 and *tps1*-21 were shown to contain single integrations of pTPSKO at the *Tps1* locus. Strains tps1-9, and tps1-13 were selected for phenotype analysis. Strains containing a single non-targeted integration of pTPSKO (ectopic integration) were found to be unsuitable for phenotypic analysis, due to highly variable behaviour (data not shown).

7.3.3 The ability of *tps1* mutants to synthesise trehalose.

Tps1 mutants were tested for their ability to synthesise trehalose during growth in culture. Strains *tps1*-9, *tps1*-13 and SN15 were grown on minimal media for 5 dpi and 20 dpi before polar metabolites were harvested, separated and analysed by GC-MS (Figure 7.10). None of the strains had any detectable trehalose by 5 dpi, but once sporulation had occurred by 20 dpi, a large amount of trehalose was detected (9.63×10^7 units) in SN15 cultures (Figure 7.10 A).

Figure 7.8

Figure 7.9

Figure 7.10

The *tps1* mutant *tps1*-9 did not produce any detectable trehalose, even at 20 dpi. *tps1*-13 was found to have a very low amount of trehalose at 20 dpi, (3.9×10^5 units), only 0.4 % of wild-type levels and marginally above the limits of detection. In addition, glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) levels were measured in the same cultures. Both *tps1*-9 and *tps1*-13 contained significantly more G6P than SN15 at 5 dpi, by 20 dpi G6P was almost totally absent in all cultures (Figure 7.10 B). Average F6P abundance in *tps1*-9 was larger than in SN15, but was not significantly different, while *tps1*-13 contained similar amounts to SN15 (Figure 7.10 C).

Trehalose levels were also quantified after infection of wheat with each *tps1* mutant. A detached leaf assay (DLA) was performed with each strain and polar metabolites extracted at 7 dpi, for analysis by GC-MS (Figure 7.11). The wild-type strain SN15 produced large amounts of trehalose *in planta* (6.2×10^7 units), while the mock infected sample contained 5.3×10^5 units of trehalose. The relative amounts of trehalose relative to SN15 were as follows; Mock, 0.9%; *tps1*-9, 0.6%; *tps1*-13, 0.9%. The mutants lacking *Tps1* showed an almost total loss of trehalose during both *in vitro* and *in planta* growth situations.

To check whether spores were a significant store of trehalose within the strains, pycnidiospores were harvested from cultures grown on a complete medium (CZV8CS) and on a defined medium that lacked trehalose (MM), the trehalose content was quantified using GC-MS in SIM-mode (Figure 7.12). SIM-mode is approximately 20-fold more sensitive than scan mode and allowed detection of trehalose in small quantities of tissue.

Figure 7.11

Figure 7.12

Trehalose quantitation standards were run simultaneously to allow the calculation of definitive nanogram amounts of trehalose per mg of spores. When grown on complete media, SN15 contained over 100-fold more trehalose/mg spores than either of the mutants. The amount of trehalose for each strain grown on CZV8CS was as follows; SN15, 117.58 ng/mg; *tps1*-9, 0.97 ng/mg; *tps1*-13, 1.59 ng/mg. When grown on MM sucrose, the amount of trehalose per mg spores was as follows; SN15, 35.39 ng/mg; *tps1*-9, 5.35 ng/mg and *tps1*-13, not detected. These results showed a reduction in trehalose content in the *tps1* mutants.

7.3.4 Ability of *tps1* strains to utilise various nutrient sources.

Since the *tps1* mutants had been shown to have significantly reduced trehalose levels under different growth conditions, the ability of the mutants to utilise various carbon and nitrogen sources was measured (Figure 7.13). Defined liquid media were inoculated with pycnidiospores and incubated at 20ºC for 7 days. Sole carbon-sources used were glucose, fructose, sucrose, mannitol, trehalose, glutamate and casamino acids. Sole nitrogen sources used were, nitrate, glutamate, and casamino acids. Growth was equivalent to MM sucrose in all media except; mannitol + nitrate and glutamate alone. While there were a few slight differences in average growth rates, none of the mutants behaved significantly differently to SN15 under the conditions tested. The physical appearance and growth behaviour of the *tps1* strains was examined after growth on defined and complete solid media, and measurement of their growth rate. MM sucrose, MM sucrose + trehalose and CZV8CS solid media were spot inoculated with 5×10^5 spores in the centre of each agar plate and incubated at 20ºC until growth had almost filled the plate. Growth on the three media revealed much the same trends as seen in the liquid culture growth assays (Figure 7.14). The cultures all looked similar within the usual variation of replicate subcultures.

Figure 7.13

Figure 7.14

Colony pigmentation and production of aerial hyphae was similar across all media and strains. On MM + sucrose the mutants all grew to the same extent while SN15 grew more, as judged by the Tukey-Kramer statistical significance test (Figure 7.15). When grown on MM + sucrose + trehalose, the growth difference between SN15 and the mutant strains was reduced somewhat and on CZV8CS, the difference was no longer statistically significant. While the observed differences between SN15 and mutant strains when grown on MM + sucrose are statistically significant, if the growth of each strain was individually compared across the media, they individually did not change significantly over those three media.

7.3.5 The ability of *tps1* strains to cause disease.

Once it was established that growth behaviour of the *tps1* strains was not grossly affected on defined media, behaviour on the wheat host was tested. The ability of *tps1*-9 and *tps1*-13 to cause disease on wheat cv. Amery was tested by whole plant spray. Two-week old plants were sprayed with pycnidiospores from the mutant strains, wild-type SN15 and a mock inoculum. All of the *tps1* mutants produced lesions on two-week-old wheat plants to a comparable degree to the wild-type (Figure 7.16). Lesion formation was also tested using the detached leaf assay. Detached leaves from 2-week-old plants were spot inoculated with 5,000 spores in replicates. Lesion formation was slightly reduced by loss of the *tps1* gene as the three mutants produced lesions ~75% of the size produced by wild-type (Figure 7.17). The reduced lesion size of *tps1*-9 and *tps1*-13 was significantly less then the SN15 lesion size by a Tukey-Kramer test.

Figure 7.15

Figure 7.16

Figure 7.17

7.3.6 The ability of *tps1* strains to sporulate.

Lesion development in the *tps1* mutants had been shown to be unaltered *in vitro* and *in planta*. Sporulation was also examined to determine how the loss of *Tps1* affected the ability of *S. nodorum* to complete the asexual cycle *in vitro* and *in planta*.

7.3.6.1 Pycnidia formation during latent period assay.

The latent period is defined as the time taken for an infected leaf from a whole plant spray to produce 50 Stage 3 or Stage 4 pycnidia, after being detached and mounted in agar. The assay showed the latent periods for the two mutants were not significantly different from SN15 (Figure 7.18). To further characterise sporulation *in planta*, pycnidiospore numbers were quantified in the same leaves. The latent period assay leaves were immersed in a Tween solution (0.01%) and gently agitated to release spores from any mature pycnidia. Spores were harvested and counted on a per leaf basis. The assay showed that, in contrast to pycnidia formation, SN15 produced significantly more spores than either of the *tps1* mutants (Figure 7.19). The largest difference in spore production was seen between SN15 and *tps1*-13, with $6.7x10^4$ and $3.5x10^4$ spores/leaf, respectively.

7.3.6.2 Pycnidia formation during a detached leaf assay.

Counting the amount of Stage 3 or 4 pycnidia produced by the end of a detached leaf assay showed that the mutants lacking *Tps1* were severely affected in their ability to produce sporulation structures (Figure 7.20 A). SN15 produced an average of 61 pycnidia per leaf, while *tps1*-9 and *tps1*-13 produced an average of 20 and 19 pycnidia per leaf, respectively (Figure 7.20 B). In the absence of *Tps1* only 30 to 33 % of the wild-type number of pycnidia were produced. SN15 had significantly more pycnidia per leaf as judged by the Tukey-Kramer significance test.

Figure 7.18

Figure 7.19

Figure 7.20

While the ability to produce pycnidia was not completely eliminated in the *tps1* strains, the few pycnidia that were produced appeared smaller and did not release spores onto the leaf surface as *Tps1* strains did. While development of a pycnidium is required to begin the formation of spores, it is not necessarily *sufficient* for spore production. With this thought in mind, spores were isolated and counted from cultures grown in defined media *in vitro* for comparison with those produced *in planta*.

7.3.6.3 Sporulation during *in vitro* growth.

To measure rates of sporulation *in vitro*, *tps1* strains were grown on three media, MM sucrose, MM sucrose + trehalose, and CZV8CS. Plates were spot inoculated with 5×10^5 spores and incubated at 20 ºC for 3 weeks. Spores were harvested and counted. When grown on MM sucrose, SN15 produced approximately 10-fold more spores than the *tps1* strains (Figure 7.21). Each of the *tps1*-mutants had statistically significant reductions in spore number, both grouping apart from SN15 by the Tukey-Kramer test. When grown on MM + sucrose + trehalose, the results followed a similar pattern, with SN15 producing far more spores than any of the other strains tested. MM sucrose+trehalose medium produced the lowest spore counts for all strains. When grown on complete media (CZV8CS) all strains produced greater amounts of spores than in the other two media, but the *tps1* strains showed the greatest increase, up 22-fold for *tps1*-9 and up 5-fold for *tps1*-13.

7.3.7 Pycnidia morphology during growth *in planta*.

To determine when the *tps1* sporulation defect occurred *in planta*, detached-leaf assay leaves were stained with Trypan blue. In these examples fungal structures were stained blue and plant cells were mostly decolourised.

Figure 7.21

SN15 infections contained many pycnidia, which stained as large, opaque, spherical structures (Figure 7.22). In addition to densely stained pycnidia, widespread staining of hyphae within the leaf was visible in all SN15 examples. Samples of *tps1*-9 infections were almost completely free of pycnidia; while the *tps1*-13 sample revealed the presence of many immature pycnidia. The average pycnidia size in SN15 infections was approximately 130 µm, while in *tps1*-13 pycnidia were smaller at approximately 75 µm, statistically different by Tukey-Kramer test (alpha=0.01) (data not shown). The *tps1*-9 samples very rarely contained pycnidial primordia with distinct margins, and more commonly, hyphal knots and early-stage pycnidial development structures, but no stage 4 pycnidia were found. The immature pycnidia present in *tps1*-9 and *tps1*-13 could not be distinguished from leaf tissue under stereomicroscopy of unstained entire samples, while those in the SN15 sample were noticeable as dark punctate structures (data not shown).

Hyphal development throughout the leaf was unaffected in the *tps1* mutants; full colonisation of the leaf interior was seen in all *tps1* strains (data not shown).

7.3.8 Stress response in the *tps1* strains.

Tps1 mutants have been shown to sporulate less than the wild-type strain *in planta*. This phenotype could be due to stresses placed on the pathogen during infection. To investigate the ability of the *tps1* mutants to cope with heat stress, a spore germination assay was set up to test the ability of spores to germinate within 6 hours of deposition at 20ºC, 30ºC, 35ºC and 37ºC (Figure 7.23). All of the strains had a maximum germination ratio at either 20 or 30ºC, which then dropped markedly at 35ºC, and was always non-permissive for germination at 37ºC. Only 35ºC revealed significant differences in average germination rates between strains. At 35ºC, SN15 (29.2 %) had a

significantly higher germination ratio than either *tps1*-9 (11.8 %) or *tps1*-13 (6.7 %). There were not any significant differences in germination between strains at the other temperatures.

Since the *tps1* strains had shown a temperature-specific reduction in germination, the steady-state growth rate at permissive and semi-permissive temperatures was examined. Strains were grown for 7 days in liquid culture at 20ºC, 30ºC and 35ºC. In order to test if exogenous trehalose could complement a growth defect, two defined media were used, MM Sucrose and MM Trehalose. Growth under continuous heat stress in MM sucrose showed the *tps1* strains were more sensitive to heat than SN15 at 30ºC (Figure 7.24). At a confidence level of 0.01, the difference was statistically reliable for SN15 and *tps1*-9 but not for *tps1*-13. In MM trehalose, the growth rates were no longer significantly different. The media had an effect on the growth rate of the cultures independent of incubation temperatures. At 20ºC all MM sucrose cultures grew almost twice as fast as those grown on MM trehalose, while at 30ºC the MM sucrose cultures had slowed to the rate of the MM trehalose cultures. At 35ºC, growth was almost completely abolished in all cultures. The analysis method normalised growth for each strain and media to remove inoculum or media bias.

7.3.9 The effect of oxidative stress on *tps1* strains.

An oxidative burst is commonly used by plants to defend against infection. *Tps1* mutants were subjected to oxidative stress in liquid culture and their growth measured. The growth media was either MM sucrose or MM sucrose+trehalose containing 0 mM, 0.03 mM, or 0.1 mM t-butyl-peroxide as the oxidant. Growth on each media without t-butyl-peroxide added was recorded as 100 %, the growth in subsequent t-butyl-peroxide concentrations was recorded relative to that.

Figure 7.22

Figure 7.23

Figure 7.24

Using this method, *tps1* cultures grown in the presence of 0.03 mM and 0.01 mM t-butyl-peroxide grew more slowly in MM sucrose than wild-type cultures (Figure 7.25). The two *tps1* mutants had over twice the reduction in growth compared to SN15. When grown in MM sucrose+trehalose, the *tps1* mutants were affected by 0.03 mM t-butyl-peroxide more than SN15, but in 0.1 mM t-butyl-peroxide they were no longer significantly different than the reduction in SN15 in the same medium. The largest differences were seen at 0.01 mM t-butyl-peroxide, when trehalose was included, the effect of the oxidant was reduced by approximately half in all strains, but the *tps1* strains made the largest recovery.

7.3.10 Nitrate utilisation by *tps1* strains.

Nitrate utilisation by *S. nodorum* in relation to trehalose biosynthesis is of interest since Foster *et. al*. (2003) reported that a *Magnaporthe grisea* mutant lacking the *Tps1* gene could not utilise nitrate when glucose was the sole carbon source. Because of this, the *tps1* strains were tested for the ability to grow on various sole nitrogen sources. Four solid media were tested, minimal media with 10 mM glucose as the sole carbon source and 25 mM nitrate, nitrite, glutamate or ammonia added as sole nitrogen sources. Agar plates were spot inoculated with 5,000 spores and grown at 20ºC for one week (Figure 7.26). The test showed that all of the strains grew without hindrance on MM Glucose-Nitrate, MM Glucose-Glutamate and MM Glucose-Ammonia. None of the strains were able to grow on MM Nitrite. This showed that in *S. nodorum*, lack of the *Tps1* gene did not affect growth on these nitrogen sources.

Figure 7.25

Figure 7.26

7.4 Discussion

In chapters 5 and 6, it was found that trehalose was an abundant metabolite, strongly associated with sporulation of *S. nodorum* during growth *in planta* and *in vitro*. Trehalose accumulated during the late-infection stage *in planta*, and correlated with the onset of sporulation both *in planta* and *in vitro*. Trehalose was also found to be a major constituent of the pycnidiospore, at greater concentrations than that found in samples of an entire culture. Using sequence similarity to characterised trehalose metabolism genes, a candidate was identified in the *S. nodorum* genome sequence, likely to encode a trehalose 6-phosphate synthase, it was named *Tps1*. *Tps1* was deleted from the SN15 genome by targeted gene replacement. Mutants lacking *Tps1* almost completely lost the ability to accumulate trehalose during growth *in vitro* and *in planta*. Sugar-phosphate levels were increased in *Tps1* mutants during early growth with sucrose as the sole carbon source. Pycnidiospores harvested from *tps1* strains contained greatly reduced trehalose levels compared to spores from SN15. While the ability to cause lesions on wheat was only slightly affected by the loss of *Tps1*, sporulation was greatly reduced both *in vitro* and *in planta*. The ability to resist heat and oxidative stress was reduced in *Tps1* mutants under certain situations *in vitro*.

7.4.1 Identification of a gene potentially encoding a trehalose 6-phosphate synthase.

The *S. nodorum* genome sequence was found to contain a close homologue of the *S. cerevisiae Tps1* gene. A ClustalW alignment of fungal *Tps1* amino acid sequences showed that *S. nodorum* SNOG_09603 was very similar to previously characterised fungal *Tps1* genes. In addition it was shown that although members of the trehalose pathways in fungi are similar at a sequence level, SNOG_09603 was most likely to

encode a Tps1. When compared to the *Emericella nidulans* annotated protein set, SNOG-09603 and EnTps1 were reciprocal best hits in a blastP analysis. Four other genes were identified in the *S. nodorum* genome that were likely to encode the other related members of trehalose metabolism; trehalose 6-phosphate phosphorylase (SNOG_02747), trehalose regulatory subunit (SNOG_03369), neutral trehalase (SNOG_01591) and Tre1 trehalase (SNOG_12745). Therefore, SNOG_09603 was the most likely candidate for a Tps1 encoding gene, and was not more closely related to another trehalose biosynthesis gene. Incidentally, SNOG_12745 shares a high degree of similarity with other Tre1 homologues, yet is the only example of this type outside of the *Sordariomycetes*.

S. cerevisiae trehalose biosynthesis and degradation enzymes share a high degree of sequence similarity, approximately 30% at the amino acid level. *S. nodorum* was found to have even higher similarity between the putative members of the same pathway, SnTps1 shared between 37% and 48% similarity at the aa level with *S. nodorum* Tps2, Tps3, Nth1 and Tre1 candidates. This suggests that the *S. nodorum* trehalose enzymes may be co-localised in a complex, as reported in yeast.

7.4.2 Deletion of *Tps1* in *S. nodorum*.

Targeted gene replacement was used to replace the Tps1 gene with the phleomycin resistance cassette. Two mutants, *tps1*-9 and *tps1*-13, were shown to contain a single integration event at the Tps1 locus. Three strains were identified as ectopic mutants, containing an integrated construct and an intact *Tps1* locus. These mutants were checked for their suitability as control strains during phenotypic characterisation of *tps1*-9 and *tps1*-13; they were found to have a variable phenotype and were unable to be used as control strains. While the Southern blot indicated they each contained an intact

Tps1 gene, *Tps1*-1 and *Tps1*-3 were likely to contain multiple insertions of the construct, as indicated by the greater signal intensity from the 1.5 kb hybridising fragment compared to the 0.9 Kb wild-type fragment. The variable phenotype of the ectopic strains may have arisen due to gene silencing of the Tps1 locus. The knockout construct did not contain any *Tps1* coding sequence, but regions homologous to the 5' and 3' untranslated regions (UTRs) were present. This may have been enough to trigger silencing, causing an unpredictable phenotype. While an ectopic insertion mutant is often used as a control strain during phenotypic characterisation, it is not as reliable a control as one with full genetic complementation. Non-homologous integration events may interfere with other gene's function, with unknown effects. The *tps1*-9 strain was selected for genetic complementation, to restore the *Tps1* locus at another genomic location. Unfortunately, this was not achieved within the time constraints of the study, but will be completed for future studies of the *tps1* mutants.

7.4.3 Strains lacking the *Tps1* locus do not accumulate trehalose to wild-type levels.

Trehalose levels in strains lacking the *Tps1* locus were determined during growth *in planta* and *in vitro*. *In planta*, trehalose levels were less than 1% of wild-type levels in *tps1*-9 and *tps1*-13. *In vitro,* trehalose could not be detected in *tps1*-9 and was 0.4% of wild-type levels in *tps1*-13 on MM sucrose at 20 dpi. In spores, trehalose content was 7 fold less in *tps1*-9 when grown on MM sucrose, and was over 100-fold less when grown on CZV8CS complete medium. Overall, this showed that the amount of trehalose in cultures was correlated with the presence of the *Tps1* locus.

Trehalose was not completely absent from cultures in *tps1* strains, which was not unexpected. Deletion of *Tps1* in *M. grisea* did not completely remove trehalose from

mycelial samples (Foster *et al.*, 2003), while Doehlemann *et. al.* (2006) reported traces of trehalose remained in conidia of a *Botrytis cinerea tps1* strain. It is possible that other disaccharide synthesising enzymes have non-specific activities and may produce a small amount of trehalose, or trehalose-like sugars. The GC-MS technique used to quantify trehalose may confuse low-abundance disaccharides if they have very similar retention times. There are two other trehalose biosynthetic pathways described in the literature other than the OtsA pathway typical of fungi. The TreY-TreZ and the TreS pathways produce trehalose from glycogen and maltose, respectively, and are most commonly found in the mycobacteria. Close homologues for genes encoding these three enzymes were not found in the *S. nodorum* genome. It is most probable that the residual trehalose present in *tps1 strains* was scavenged from the environment, or was produced via non-specific disaccharide synthases.

In addition to a reduction in trehalose content, *tps1* strains accumulated both fructose 6-phosphate and glucose 6-phosphate. These glycolytic intermediates have been observed to accumulate in yeast (Van Aelst *et al.*, 1993) and in *A. nidulans tps1* strains (Fillinger *et al.*, 2001). While yeast mutants suffered from depleted ATP levels and were unable to grow on glucose, *A. nidulans* mutants did not have reduced ATP and could utilise both glucose and fructose as carbon sources. ATP levels were not quantified in the *S. nodorum tps1* strains, but their utilisation of glucose and fructose is similar to the *A. nidulans* mutants..

7.4.4 *tps1* strains have markedly reduced sporulation.

The most noticeable effect of the *Tps1* deletion was a large reduction in the development of pycnidia and the spores within them. Sporulation of the *tps1* strains was measured during growth *in planta* and *in vitro*, and was reduced in both situations.

The number of pycnidia produced in a DLA at 7 dpi by *tps1* strains was only 30% of the wild-type level. A latent period assay (LPA) showed that pycnidia were accumulating at a similar rate in *tps1* and wild-type strains, but when spores were harvested from the same leaves, the spore counts were significantly reduced. This indicated that while pycnidia may have formed to a similar extent in a LPA, the numbers of mature spores within them was reduced.

The discrepancy in pycnidia formation in the DLA and LPA assays of the *tps1* mutants may be accounted for by the differences in infection methodologies. A DLA uses a large number of spores spot inoculated onto a 2-week-old detached wheat leaf. A LPA uses a spore solution sprayed over entire 2-week-old plants, disease is allowed to progress for a week before the leaves are detached to allow sporulation to occur. The LPA gives the pathogen far more time and more of the leaf surface to attack, while the DLA covers a shorter period and has a single point of infection. The formation of pycnidia in *tps1* strains appears to be quite finely balanced between success and failure, as indicated by the DLA and LPA results. This study did show however, that pycnidia formation was not sufficient for spore formation, as the pycnidia formed during the LPA did not release the expected number of spores. This result ties in with the observed enrichment of trehalose in the wild-type pycnidiospore, only when the spores are being formed does the lack of *Tps1* become critical. The reduction in pycnidia formation observed in the DLA may be due to the more intense infection situation putting more strain on the pathogen. The DLA contains more necrotic tissue, with an associated production of polyphenolic compounds by the plant. The necrotic leaf environment is probably far more hostile to the invading pathogen in a DLA, resulting in less pycnidia formation.

Pycnidia formed during the *tps1*-9 and *tps1*-13 DLAs were found to be smaller than usual. They appeared to be arrested at the stage of 'immature pycnidia', which is when the internal hyphae begin to differentiate into conidiogenous cells (Douaiher *et al.*, 2004). The immature pycnidia were not visible in unstained samples, indicating that melanisation had not yet occurred. Perhaps accumulation of trehalose is a developmental checkpoint for melanin formation in pycnidia.

Sporulation was also reduced during growth *in vitro*. Spore counts were significantly lower in *tps1* mutants when grown on defined media, with or without trehalose added. This was a clear demonstration that addition of exogenous trehalose could not complement the reduced-sporulation phenotype. This is similar to the *tps1* strain of *M .grisea* where a pathogenicity defect was not able to be restored by addition of exogenous trehalose (Foster *et al.*, 2003). The sporulation of wild-type and *tps1* strains was not significantly different when grown on CZV8CS complete medium. This was expected, as *S. nodorum* sporulates very well when grown on CZV8CS. CZV8CS does not contain any added trehalose, but is 20% (v/v) vegetable juice (V8 brand), an undefined component. V8 juice was analysed by GC-MS to see if trehalose was present in significant quantities, none was detected (data not shown). It appears that CZV8CS can increase the sporulation from *tps1* strains, but in a manner independent of trehalose. The inability of exogenous trehalose to complement the sporulation phenotype correlates with the observation that trehalase enzymes have been observed to localise to the cell wall in *N. crassa (Thevelein, 1984)* . If present, they would hydrolyse external trehalose to glucose prior to or soon after transport into the cell, this would prevent accumulation of imported trehalose in the cell. Indeed, *S. nodorum* was shown in this

study to grow more slowly on trehalose as a sole carbon source, when compared to growth on sucrose. Perhaps this is an indication that the uptake and/or degradation of trehalose from the environment is limited, and certainly less than degradation of sucrose.

7.4.5 *Tps1* is dispensable for lesion formation by *S. nodorum*

Tps1 mutants were shown to form lesions to an extent comparable with wild-type. Lesion formation was determined by whole plant spray (WPS), which showed that the pathogenicity, or lesion formation was not statistically different to that of SN15 on entire seedlings. Lesion formation during the DLA was significantly reduced, but to a small degree. Lesions on DLAs were approximately 75% of the size of those produced by SN15. The small reduction in lesion formation may be caused by reduced response to plant defense. As mentioned earlier, the DLA is a shorter, more testing assay for infection, compared to the WPA or LPA. It may be that trehalose helps *S. nodorum* colonise new tissue, but is certainly not essential for penetration of the leaf cuticle, lesion development or colonisation of the leaf interior. This phenotype is similar to that described for *B. cinerea tps1,* in which lesion development was not affected at all, but different to *M. grisea* where *Tps1* was required for cuticle penetration via appressoria (Doehlemann *et al.*, 2006; Foster *et al.*, 2003). In *Cryptococcus neoformans*, a fungal pathogen of mammals, *Tps1* was shown to be required for virulence on rabbits, mice and on the invertebrate *Caenorhabditis elegans*. It appears that *Tps1* is required for many different roles in different organisms, even within fungal ascomycetes.

7.4.6 Strains lacking *Tps1* are more sensitive to external stresses.

Defects in both sporulation and lesion development were observed in *tps1* mutants. These deficiencies may be due to inadequate stress responses. Oxidative and heat stress

are both commonly associated with trehalose presence in fungi, for this reason both stresses were examined in the *tps1* background. *Tps1* strains exhibited increased sensitivity to heat stress during germination and growth in liquid culture. At 35ºC *tps1* pycnidiospores were approximately 50% less likely to germinate than SN15 spores. During steady-state growth at high temperatures, *tps1* strains grew less than SN15 at 30ºC, while the growth difference was cancelled when trehalose present in the growth medium. The increase in sensitivity was only marginally significant by the Tukey-Kramer test. The addition of trehalose removed the difference in growth rate between SN15 and the *tps1* mutants, this was in contrast to the *tps1* sporulation defect, which couldn't be rescued by exogenous trehalose. This contradictory situation may be explained by the mode of action of trehalose and heat stress. It has been shown that trehalose is highly likely to interact with lipid bilayers, and that lipid stability is a key factor in survival of heat stress (Doxastakis *et al.*, 2005; Elbein *et al.*, 2003). It may be possible that external trehalose can bind to and stabilise the external cell membrane, thereby reducing lipid instability and heat-induced damage without having to be transported into the cell. This theory is compatible with the observed inability of exogenous trehalose to repair *tps1* sporulation defects, which would require transport of trehalose into the cell.

The ability of the *tps1* strains to resist oxidative stress was also investigated. *Tps1* strains were shown to be more sensitive than SN15 to oxidative stress. Growth was significantly reduced in the presence of 0.03 and 0.1 mM t-butyl-peroxide when grown in MM sucrose. In MM sucrose+trehalose medium, the effect was reduced, especially with 0.1 mM t-butyl-peroxide. Growth of SN15 was also improved by the addition of trehalose in addition to oxidative stress, but the *tps1* mutants recovered to a greater

extent. In *S. cerevisiae* it has been shown that oxygen radicals damaged amino groups on cellular proteins, and that trehalose reduced this effect. It was suggested that trehalose quenched the oxygen radicals in a similar manner to polyols, such as mannitol (Benaroudj *et al.*, 2001). The presence of millimolar concentrations of trehalose in the growth medium may greatly reduce the free radical concentration.

7.4.7 Growth on rapidly fermentable carbon sources is unaffected in *tps1* mutants.

Loss of *Tps1* in *S. cerevisiae* caused mutants be become unable to grow on sole-carbon sources such as glucose or fructose (Noubhani *et al.*, 2000; Thevelein and Hohmann, 1995). The postulated mechanism suggests that trehalose 6-phosphate is a key regulator of glycolysis in yeast. Loss of *Tps1* resulted in uncontrolled entry of glucose into the glycolytic pathway, accumulation of sugar phosphate glycolytic intermediates, depletion of intermediates after glyceraldehyde-3-phosphate dehydrogenase, which lead to exhaustion of cellular ATP and a halt to growth. Growth of *S. nodorum tps1* strains on several defined media revealed that they were unaffected in their ability to utilise glucose, fructose and sucrose, although they did accumulate glucose 6-phosphate during initial growth on sucrose. A glucose non-utilising phenotype was initially described for a *M. grisea tps1* mutant (Foster *et al.*, 2003), however the true limitation was later found to be that Tps1 activity was essential for nitrate reductase activity, and not for regulation of glycolysis (Wilson and Talbot, 2006). *S. nodorum Tps1* mutants were grown on minimal media with various simple nitrogen sources to determine if they were nitrate non-utilising. *Tps1* was shown to be dispensable for nitrate, glutamate and ammonia utilisation. No growth was observed for any strains on nitrite, which was an expected outcome as nitrite is usually toxic for fungi.

7.4.8 Summary

Tps1 has been shown to be required for accumulation of trehalose to high levels during growth in culture and *in vitro*. Loss of *Tps1* was dispensable for lesion formation but caused a reduction in sporulation both *in planta* and *in vitro*. The reduction in sporulation may be due to the increased sensitivity of *tps1* strains to oxidative and heat stress. Trehalose appears to be a crucial sporulation metabolite and may have roles in stress defence in *S. nodorum*.

Chapter 8

Final discussion

8.1 *Stagonospora nodorum* as a model necrotroph

Necrotrophic fungal pathogens have a significant impact on agriculture across the world. Control strategies using fungicides are usually based on prevention of the pathogen spores from germinating, or once infection has occurred, from colonising the host tissue (Hewitt, 1998). While targeting these aspects of the life cycle of pathogens has undoubtedly been effective, a third part of the lifecycle, sporulation, remains largely untouched. Here, *Stagonospora nodorum* was used as a model necrotroph for the elucidation of the requirements for sporulation of fungal plant pathogens. *S. nodorum* is well suited to study as it is amenable to molecular genetic techniques, has a rapid disease cycle and is a significant pathogen of commercial wheat crops (Solomon *et al.*, 2006c). The damaging disease, leaf and glume blotch only occurs when several rounds of asexual reproduction occur, within a small area around the plant in question. In Western Australia, wind-borne ascospores release peaks during the seedling to early flag emergence stages, suggesting the asexual cycle is required for the majority of grain damage (Bathgate and Loughman, 2001). If several rounds of asexual sporulation do not occur in rapid succession, the infection is left behind on the lower plant architecture, a less costly infection. This suggests that a complete block of asexual sporulation would greatly reduce damage to the grain, even while allowing unrestricted filamentous growth from air borne ascospores. The genetic requirements for asexual sporulation of *S. nodorum* were investigated in two different ways, from transcriptional and metabolic perspectives.

8.2 Overview of key findings

The transcriptional approach, comparing two cDNA libraries, one derived from *in vitro* and one from *in planta* growth, revealed broad changes in gene expression. The main differences in transcript abundances between the two libraries related to the nutrient source of the respective cultures, with the *in planta* library containing more transcripts related to complex carbohydrate catabolism and proteolysis, and the *in vitro* library containing more transcripts related to lipid metabolism. The transcriptional activity of several genes was confirmed, by quantitative PCR, to be greatly upregulated during *in planta* growth and also during sporulation. These included genes similar to an arabinofuranosidase, an aldose 1-epimerase, an oxidoreductase, an arabitol 4-dehydrogenase and a norsolorinic acid reductase. It was interesting to note that three of the upregulated genes were involved in carbohydrate metabolism. Targeted gene replacement showed that the *Abd1* gene orthologous to an arabitol 4-dehydrogenase was required for full asexual sporulation during growth *in planta*. A reduction of at least 20 % was seen in each of three *abd1* knock-out strains. Arabitol was not able to be conclusively identified by GC-MS and was unable to be resolved from xylitol. Even so, in the *in vitro* metabolomics study, the combined arabitol/xylitol abundance was observed to increase with sporulation. Analysis of pycnidiospores showed arabitol/xylitol was the fourth most abundant metabolite, comprising 6 % of the total peak area, while mannitol comprised 38 % of the total area. These results showed that even minor polyols found in *S. nodorum* may have significant contributions to the overall fitness of the organism.

The metabolomics approach focussed on primary metabolism during sporulation *in planta* and *in vitro*. The main metabolic shifts that were observed to correlate with

sporulation were those related to carbohydrate metabolism, in particular the accumulation of mannitol and trehalose during sporulation in both *in planta* and *in vitro* growth conditions. Mannitol and trehalose were shown to be the major constituents of the pycnidiospore, comprising over 50 % of the total peak area when analysed by GC-MS. Metabolites in the TCA cycle, fumaric acid, malic acid and succinic acid, were observed to increase in abundance as sporulation occurred, in conjunction with an increase in apolar compounds.

The link between trehalose biosynthesis and sporulation was confirmed by a reverse-genetics approach. A targeted knockout of the *Tps1* gene encoding a trehalose 6-phosphate synthase, showed it was required for full accumulation of trehalose, with *tps1* strains almost completely lacking trehalose. The *tps1* strains had markedly reduced ability to sporulate asexually, with both pycnidia and pycnidiospore formation affected *in planta* and on defined media. The loss of *Tps1* also resulted in an increased sensitivity to heat and oxidative stress.

8.3 Transcriptomics versus metabolomics

This study affords a perspective on the comparison of transcript-based and metabolite-based high-throughput technologies. Here, both transcripts and metabolite abundance were correlated with growth *in planta* and *in vitro*, covering sporulating and non-sporulating developmental states.

It was interesting to note that both approaches highlighted almost entirely different aspects of the pathogens biology. For example, while mannitol and trehalose represented a large proportion of the metabolite pool during growth *in planta* and *in*

vitro, transcripts from the key enzymes involved were exceedingly rare. For instance, of the five genes predicted to be involved in trehalose biosynthesis and breakdown in *S. nodorum*, *Tps1, Tps2, Tps3* and *Tre1* and *Nth1*, none were represented in either cDNA libraries. Genes involved in mannitol metabolism, such as those encoding mannitol 1-phosphate dehydrogenase *Mpd1* (SNOG_12666) and mannitol dehydrogenase *Mdh1* (SNOG_15488) were represented in both libraries, but at low levels. ESTs assigned to the two genes were as follows, *Mpd1; in planta* 6, *in vitro* 0, *Mdh1; in planta* 1 *in vitro* 8. While some transcription was observed, it was almost completely divergent, with each gene expressed in either *in planta* or *in vitro*, but not both. *S. nodorum Mpd1* expression during a detached leaf assay has been shown to be approximately 2-fold more than *Mdh1 in planta* (Solomon *et al.*, 2006e). These previously reported expression levels were consistent with the *in planta* cDNA library, the lack of *Mdh1* transcripts can be explained by the poor sensitivity of cDNA library analysis.

Combined transcriptomics and metabolomics studies have been performed previously. A parallel study using both transcriptomics and metabolomics to differentiate developmental stages of potato tuber found that metabolite abundances were able to resolve various tissue types with far greater accuracy than transcripts were able to (Urbanczyk-Wochniak *et al.*, 2003). While a significant number of correlations were observed, they were not usually between genes and metabolites within the same pathway. A combined transcriptomics and metabolomics study of the *Arabidopsis* response to nitrogen and sulphur starvation showed the genes involved in glucosinolate metabolism were shown to be co-ordinately regulated with metabolite abundance (Hirai *et al.*, 2004). However, there was not a common link between transcription of genes, translation of mRNA and production of a metabolite. While transcript and metabolite

abundances do not necessarily correlate well, transcript and protein abundance generally do so to a greater degree. A study of protein and mRNA levels in yeast showed that protein abundance in 15 of 289 examples was regulated at a post transcriptional level (Ideker *et al.*, 2001). It is a reasonable assumption that mRNA and protein abundances may correlate, however metabolite abundances are subject to even more regulatory mechanisms, and are not as likely to maintain a correlation with transcription.

Overall, this study has shown that transcriptomics and metabolomics are both highly informative techniques for analysis of developmental phenomena. Although both methods are useful, they can highlight different aspects of cellular biology, and as such are best used as complementary techniques.

8.4 Future directions

Asexual sporulation in *S. nodorum* has been shown to be affected by the loss of two genes, involved in trehalose and arabitol biosynthesis. The contribution of each to sporulation has not yet been defined, therefore future work should attempt to address this. The role of trehalose in sporulation was linked to the enrichment of trehalose in the pycnidiospore. Whether trehalose is acting as a storage carbohydrate, anti-oxidant or osmo-protectant is unknown. Mutagenesis of the remaining members of the trehalose biosynthetic and degradation pathways would help to determine which activities are required, at each stage of development. In particular, it may help determine the cellular location of the enzymes. Work in *M. grisea* showed a showed that *Tps1* was required for appressorium function. Considering *S. nodorum* does not require such specialised infection structures for penetration, it would be interesting to see where *S. nodorum Tps1* is first expressed during the infection.

The effect of both oxidative and heat stress on sporulation was not examined in this study. The clear reduction in sporulation of *tps1* strains may become even more pronounced at semi-permissive heat and oxidative conditions. These aspects could be easily tested by growing the *tps1* strains on solid media, in the presence of various oxidative or heat stresses.

Trehalose biosynthesis may present a fungicidal target to reduce sporulation *in planta*, and hence reduce the likelihood of glume blotch in a field situation. Trehalose is commonly found in fungi and in certain "resurrection" plants (Elbein *et al.*, 2003), yet is generally not accumulated in higher plants. Multiple plant homologues for genes involved in its biosynthesis and degradation have been identified in *A. thaliana,* as well as minute amounts of a disaccharide thought to be trehalose (Muller *et al.*, 1999). Validamycin A is a potent inhibitor of trehalase activity, which has significant fungicidal activities and has been shown to cause trehalose accumulation in tobacco plants (Goddijn *et al.*, 1997; Iwasa *et al.*, 1971). While trehalose breakdown has been clearly exploited as a fungicidal target, prevention of trehalose biosynthesis could result in a more specific reduction in sporulation of fungal pathogens. A stable analogue of trehalose 6-phosphate would inhibit trehalose 6-phosphate phosphorylase, and perhaps produce a toxic accumulation of trehalose 6-phosphate. While trehalose is not usually accumulated in crop plants, any inhibition of trehalose biosynthesis would need to be carefully balanced with toxic effects on the plant itself.

8.5 Conclusion.

The project aim to isolate genes that are required for sporulation of *S. nodorum* has been fulfilled. Two genes, involved in different aspects of carbohydrate metabolism were shown to have important roles in asexual sporulation. Transcriptomic and metabolomic approaches proved to be both highly informative and complementary techniques, each highlighting different aspects of the pathogen's biology. These techniques should be suitable for the analysis of other aspects of the *S. nodorum* lifecycle, and hopefully reveal more about the general behaviour of necrotrophic fungal pathogens.

Chapter 9

Bibliography

Abebe, T., Guenzi, A.C., Martin, B., and Cushman, J.C. (2003) Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. *Plant Physiology* **131**: 1748-1755.

Adams, T.H., and Wieser, J.K. (1999) Asexual sporulation: conidiation. In *Molecular fungal biology*. Oliver, R.P. and Schweizer, M. (eds). Cambridge, UK; New York: Cambridge University Press.

Aharoni, A., Ric de Vos, C.H., Verhoeven, H.A., Maliepaard, C.A., Kruppa, G., Bino, R., and Goodenowe, D.B. (2002) Nontargeted metabolome analysis by use of Fourier Transform Ion Cyclotron Mass Spectrometry. *Omics* **6**: 217-234.

Allwood, W.J., Ellis, D.I., Heald, J.K., Goodacre, R., and Mur, L.A.J. (2006) Metabolomic approaches reveal that phosphatidic and phosphatidyl glycerol phospholipids are major discriminatory non-polar metabolites in responses by *Brachypodium distachyon* to challenge by *Magnaporthe grisea*. *The Plant Journal* **46**: 351-368.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-3402.

Arseniuk, E., Goral, T., Sowa, W., Czembor, H.J., Krysiak, H., and Scharen, A.L. (1998) Transmission of *Stagonospora nodorum* and *Fusarium* spp. on *triticale* and wheat seed and the effect of seedborne *Stagonospora nodorum* on disease severity under field conditions. *Journal of Phytopathology* **146**: 339-345.

Athenstaedt, K., Jolivet, P., Boulard, C., Zivy, M., Negroni, L., Nicaud, J.M., and Chardot, T. (2006) Lipid particle composition of the yeast *Yarrowia lipolytica* depends on the carbon source. *Proteomics* **6**: 1450-1459.

Audic, S., and Claverie, J.M. (1997) The significance of digital gene expression profiles. *Genome Research* **7**: 986-995.

Bailey, A., Mueller, E., and Bowyer, P. (2000) Ornithine decarboxylase of *Stagonospora (Septoria) nodorum* is required for virulence toward wheat. *Journal of Biological Chemistry* **275**: 14242-14247.

Baker, E.A., and Smith, I.M. (1978) Development of the susceptible and resistant reactions in wheat on inoculation with *Septoria nodourm*. *Transactions of the British Mycological Society* **71**: 475-482.

Bathgate, J.A., and Loughman, R. (2001) Ascospores are a source of inoculum of *Phaeosphaeria nodorum*, *P. avenaria f. sp. avenaria* and *Mycosphaerella graminicola* in Western Australia. *Australasian Plant Pathology* **30**: 317-322.

Bearchell, S.J., Fraaije, B.A., Shaw, M.W., and Fitt, B.D.L. (2005) Wheat archive links long-term fungal pathogen population dynamics to air pollution. *PNAS* **102**: 5438-5442.

Bell, W., Sun, W., Hohmann, S., Wera, S., Reinders, A., De Virgilio, C., Wiemken, A., and Thevelein, J.M. (1998) Composition and Functional Analysis of the *Saccharomyces cerevisiae* Trehalose Synthase Complex. *Journal of Biological Chemistry* **273**: 33311-33319.

Belocopitow, E., and Marechal, L.R. (1970) Trehalose phosphorylase from *Euglena gracilis*. *Biochim Biophys Acta* **198**: 151-154.

Benaroudj, N., Lee, D.H., and Goldberg, A.L. (2001) Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. *Journal of Biological Chemistry* **276**: 24261-24267.

Bhathal, J.S., Loughman, R., and Speijers, J. (2003) Yield reduction in wheat in relation to leaf disease from yellow (tan) spot and *septoria nodorum* blotch. *European Journal of Plant Pathology* **109**: 435-443.

Bindschedler, L.V., Sanchez, P., Dunn, S., Mikan, J., Thangavelu, M., Clarkson, J.M., and Cooper, R.M. (2003) Deletion of the SNP1 trypsin protease from *Stagonospora nodorum* reveals another major protease expressed during infection. *Fungal Genetics and Biology* **38**: 43-53.

Blazquez, M.A., Lagunas, R., Gancedo, C., and Gancedo, J.M. (1993) Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. *FEBS Letters* **329**: 51-54.

Blazquez, M.A., and Gancedo, A. (1995) Mode of action of the *gcr9* and *cat3* mutations in restoring the ability of *Saccharomyces cerevisiae tps1* mutants to grow on glucose. *Molecular and General Genetics* **V249**: 655-664.

Bloch, F. (1946) Nuclear Induction. *Physical Review* **70**: 460.

Boonsaeng, V., Sullivan, P.A., and Shepherd, M.G. (1976) Mannitol production in fungi during glucose catabolism. *Canadian Journal of Microbiology* **22**: 808-816.

Brennan, J.P., and Murray, G.M. (1998) *Economic importance of wheat diseases in Australia.* Wagga Wagga: NSW Agriculture.

Brodhagen, M., and Keller, N.P. (2006) Signalling pathways connecting mycotoxin production and sporulation. *Molecular Plant Pathology* **7**: 285-301.

Broeckling, C.D., Farag, M., Huhman, D., Naoumkina, M., Deavour, B., Lei, Z., Watson, B.S., Smith, J.T., May, G.D., Mendes, P., Dixon, R.A., Sumner, L.W., and Urbanczyk-Wochniak, E. (2005) Metabolomics and integrated functional genomics of *Medicago truncatula* responses to biotic and abiotic stress. . In *12th International Congress on Molecular Plant-Microbe Interactions* Cancun, Mexico.

Brown, D.W., Cheung, F., Proctor, R.H., Butchko, R.A., Zheng, L., Lee, Y., Utterback, T., Smith, S., Feldblyum, T., Glenn, A.E., Plattner, R.D., Kendra, D.F., Town, C.D., and Whitelaw, C.A. (2005a) Comparative analysis of 87,000 expressed sequence tags from the fumonisin-producing fungus *Fusarium verticillioides*. *Fungal Genetics and Biology* **42**: 848-861.

Brown, S.C., Kruppa, G., and Dasseux, J.L. (2005b) Metabolomics applications of FT-ICR mass spectrometry. *Mass Spectrometry Review* **24**: 223-231.

Cabib, E., and Leloir, L.F. (1958) The biosynthesis of trehalose phosphate. *Journal of Biological Chemistry* **231**: 259-275.

Calvo, A.M., Gardner, H.W., and Keller, N.P. (2001) Genetic connection between fatty acid metabolism and sporulation in *Aspergillus nidulans*. *Journal of Biological Chemistry* **276**: 25766-25774.

Calvo, A.M., Wilson, R.A., Bok, J.W., and Keller, N.P. (2002) Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews* **66**: 447-459.

Chang, P.K. (2003) The *Aspergillus parasiticus* protein AFLJ interacts with the aflatoxin pathway-specific regulator AFLR. *Molecular Genetics and Genomics* **268**: 711-719.

Chaturvedi, V., Bartiss, A., and Wong, B. (1996) Stress tolerance and pathogenic potential of a mannitol mutant of *Cryptococcus neoformans*. *Microbiology* **142**: 937-943.

Chaturvedi, V., Bartiss, A., and Wong, B. (1997) Expression of bacterial mtlD in *Saccharomyces cerevisiae* results in mannitol synthesis and protects a glycerol-defective mutants from high-salt and oxidative stress. *Journal of Bacteriology* **179**: 157-162.

Cho, E.M., Liu, L., Farmerie, W., and Keyhani, N.O. (2006) EST analysis of cDNA libraries from the entomopathogenic fungus *Beauveria (Cordyceps) bassiana*. I.

Evidence for stage-specific gene expression in aerial conidia, in vitro blastospores and submerged conidia. *Microbiology* **152**: 2843-2854.
Choi, H.K., Choi, Y.H., Verberne, M., Lefeber, A.W., Erkelens, C., and Verpoorte, R. (2004a) Metabolic fingerprinting of wild type and transgenic tobacco plants by 1H NMR and multivariate analysis technique. *Phytochemistry* **65**: 857-864.
Choi, Y.H., Tapias, E.C., Kim, H.K., Lefeber, A.W., Erkelens, C., Verhoeven, J.T., Brzin, J., Zel, J., and Verpoorte, R. (2004b) Metabolic discrimination of *Catharanthus roseus* leaves infected by phytoplasma using 1H-NMR spectroscopy and multivariate data analysis. *Plant Physiology* **135**: 2398-2410.
Clamp, M., Cuff, J., Searle, S.M., and Barton, G.J. (2004) The Jalview Java alignment editor. *Bioinformatics* **20**: 426-427.
Colebatch, G., Desbrosses, G., Ott, T., Krusell, L., Montanari, O., Kloska, S., Kopka, J., and Udvardi, M.K. (2004) Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. *Plant Journal* **39**: 487-512.
Cooley, R., Shaw, R., Franklin, F., and Caten, C. (1988) Transformation of the phytopathogenic fungus *Septoria nodorum* to hygromycin B resistance. *Current Genetics* **13**: 383-386.
Cooley, R.N., and Caten, C.E. (1991) Variation in electrophoretic karyotype between strains of *Septoria nodorum*. *Molecular and General Genetics* **228**: 17-23.
Cooley, R.N., Monk, T.P., McLoughlin, S.B., Foster, S.G., and Dancer, J.E. (1999) Gene disruption and biochemical characterisation of 3-isopropylmalate dehydrogenase from *Stagonospora nodorum*. *Pesticide science Mar*: 364-367.
Cunfer, B.M. (1999) *Stagonospora* and *Septoria* pathogens of cereals: The infection process. In *Septoria and Stagonospora diseases of cereals: A compilation of global research*. van Ginkel, M., McNab, A. and Krupinsky, J. (eds). Mexico: D.F: CIMMYT.
Czembor, P.C., Arseniuk, E., Czaplicki, A., Song, Q.J., Cregan, P.B., and Ueng, P.P. (2003) QTL mapping of partial resistance in winter wheat to *Stagonospora nodorum* blotch. *Genome* **46**: 546-554.
Davis, D.J., Burlak, C., and Money, N.P. (2000) Osmotic pressure of fungal compatible osmolytes. *Mycological Research* **104**: 800-804.
De Silva-Udawatta, M.N., and Cannon, J.F. (2001) Roles of trehalose phosphate synthase in yeast glycogen metabolism and sporulation. *Molecular Microbiology* **40**: 1345-1356.
De Smet, K.A., Weston, A., Brown, I.N., Young, D.B., and Robertson, B.D. (2000) Three pathways for trehalose biosynthesis in mycobacteria. *Microbiology* **146 (Pt 1)**: 199-208.
De Virgilio, C., Hottiger, T., Dominguez, J., Boller, T., and Wiemken, A. (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant. *European Journal of Biochemistry* **219**: 179-186.
de Vries, R.P., Flitter, S.J., van de Vondervoort, P.J., Chaveroche, M.K., Fontaine, T., Fillinger, S., Ruijter, G.J., d'Enfert, C., and Visser, J. (2003) Glycerol dehydrogenase, encoded by gldB is essential for osmotolerance in *Aspergillus nidulans*. *Molecular Microbiology* **49**: 131-141.
Desbrosses, G.G., Kopka, J., and Udvardi, M.K. (2005) Lotus japonicus metabolic profiling. Development of gas chromatography-mass spectrometry resources for the study of plant-microbe interactions. *Plant Physiology* **137**: 1302-1318.

Dijkema, C., Kester, H.C., and Visser, J. (1985) 13C NMR studies of carbon metabolism in the hyphal fungus *Aspergillus nidulans*. *PNAS* **82**: 14-18.
Doehlemann, G., Berndt, P., and Hahn, M. (2006) Trehalose metabolism is important for heat stress tolerance and spore germination of *Botrytis cinerea*. *Microbiology* **152**: 2625-2634.
Douaiher, M.N., and Halama, P. (2002) The pycnidium ontogeny of *Stagonospora nodorum,* the glume blotch agent of wheat. In *Mededelingen Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen, Universiteit Gent. 2002; 67(2): 205 208*: Ghent, Belgium: Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen.
Douaiher, M.N., Halama, P., and Janex-Favre, M.C. (2004) The ontogeny of *Stagonospora nodorum* pycnidia in culture. *Sydowia* **56**: 39-50.
Doxastakis, M., Sum, A.K., and de Pablo, J.J. (2005) Modulating membrane properties: the effect of trehalose and cholesterol on a phospholipid bilayer. *Journal of Physical Chemistry B* **109**: 24173-24181.
Dunn, W.B., and Ellis, D.I. (2005) Metabolomics: Current analytical platforms and methodologies. *Trends in Analytical Chemistry* **24**: 285-294.
Dutzmann, S., Hayakawa, H., Oshima, A., and Suty-Heinze, A. (2004) Fluoxastrobin: the leaf-systemic, broad spectrum strobilurin. *Pflanzenschutz Nachrichten Bayer* **57**: 415-435.
Ebbole, D.J., Jin, Y., Thon, M., Pan, H., Bhattarai, E., Thomas, T., and Dean, R. (2004) Gene discovery and gene expression in the rice blast fungus, *Magnaporthe grisea*: analysis of expressed sequence tags. *Molecular Plant Microbe Interactions* **17**: 1337-1347.
Eichmann, R., Biemelt, S., Schafer, P., Scholz, U., Jansen, C., Felk, A., Schafer, W., Langen, G., Sonnewald, U., Kogel, K.H., and Huckelhoven, R. (2006) Macroarray expression analysis of barley susceptibility and nonhost resistance to *Blumeria graminis*. *Journal of Plant Physiology* **163**: 657-670.
Elbein, A.D., Pan, Y.T., Pastuszak, I., and Carroll, D. (2003) New insights on trehalose: a multifunctional molecule. *Glycobiology* **13**: 17R-27.
Erdmann, R., and Kunau, W.H. (1994) Purification and immunolocalization of the peroxisomal 3-oxoacyl-CoA thiolase from *Saccharomyces cerevisiae*. *Yeast* **10**: 1173-1182.
Fiehn, O., Kopka, J., Dormann, P., Altmann, T., Trethewey, R.N., and Willmitzer, L. (2000) Metabolite profiling for plant functional genomics. *Nature Biotechnology* **18**: 1157-1161.
Fiehn, O. (2002) Metabolomics - the link between genotypes and phenotypes. *Plant Molecular Biology* **48**: 155-171.
Fillinger, S., Chaveroche, M.K., van Dijck, P., de Vries, R., Ruijter, G., Thevelein, J., and d'Enfert, C. (2001) Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*. *Microbiology* **147**: 1851-1862.
Fischer, G., Braun, S., Thissen, R., and Dott, W. (2006) FT-IR spectroscopy as a tool for rapid identification and intra-species characterization of airborne filamentous fungi. *Journal of Microbiology Methods* **64**: 63-77.
Forgue, P., Halouska, S., Werth, M., Xu, K., Harris, S., and Powers, R. (2006) NMR metabolic profiling of *Aspergillus nidulans* to monitor drug and protein activity. *Journal of Proteome Research* **5**: 1916-1923.

Forster, J., Famili, I., Fu, P., Palsson, B.O., and Nielsen, J. (2003) Genome-Scale Reconstruction of the *Saccharomyces cerevisiae* Metabolic Network. *Genome Res.* **13**: 244-253.

Foster, A.J., Jenkinson, J.M., and Talbot, N.J. (2003) Trehalose synthesis and metabolism are required at different stages of plant infection by *Magnaporthe grisea*. *EMBO Journal* **22**: 225-235.

Friesen, T.L., Stukenbrock, E.H., Liu, Z., Meinhardt, S., Ling, H., Faris, J.D., Rasmussen, J.B., Solomon, P.S., McDonald, B.A., and Oliver, R.P. (2006) Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature Genetics* **38**: 953-956.

Gadd, G.M., Chalmers, K., and Reed, R.H. (1987) The role of trehalose in dehydration resistance of *Saccharomyces cerevisiae*. *FEMS Microbiology Letters* **48**: 249-254.

Garg, A.K., Kim, J.-K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochian, L.V., and Wu, R.J. (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *PNAS* **99**: 15898-15903.

Garrett, K.A., Dendy, S.P., Frank, E.E., Rouse, M.N., and Travers, S.E. (2006) Climate change effects on plant disease: genomes to ecosystems. *Annual Review of Phytopathology* **44**: 489-509.

Giaever, H.M., Styrvold, O.B., Kaasen, I., and Strom, A.R. (1988) Biochemical and genetic characterization of osmoregulatory trehalose synthesis in *Escherichia coli*. *Journal of Bacteriology* **170**: 2841-2849.

Goddijn, O.J., Verwoerd, T.C., Voogd, E., Krutwagen, R.W., de Graaf, P.T., van Dun, K., Poels, J., Ponstein, A.S., Damm, B., and Pen, J. (1997) Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. *Plant Physiology* **113**: 181-190.

Gonzalez-Parraga, P., Hernandez, J.A., and Arguelles, J.C. (2003) Role of antioxidant enzymatic defences against oxidative stress H(2)O(2) and the acquisition of oxidative tolerance in *Candida albicans*. *Yeast* **20**: 1161-1169.

Gooding, M.J., Dimmock, J.P.R.E., France, J., and Jones, S.A. (2000) Green leaf area decline of wheat flag leaves: the influence of fungicides and relationships with mean grain weight and grain yield. *Annals of Applied Biology* **136**: 77-84.

Griffin, T.J., Gygi, S.P., Ideker, T., Rist, B., Eng, J., Hood, L., and Aebersold, R. (2002) Complementary profiling of gene expression at the transcriptome and proteome levels in *Saccharomyces cerevisiae*. *Molecular and Cellular Proteomics* **1**: 323-333.

Gronover, C.S., Kasulke, D., Tudzynski, P., and Tudzynski, B. (2001) The role of G protein alpha subunits in the infection process of the gray mold fungus *Botrytis cinerea*. *Molecular Plant-Microbe Interactions* **14**: 1293-1302.

Gygi, S.P., Rochon, Y., Franza, B.R., and Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* **19**: 1720-1730.

Halama, P., and Lacoste, L. (1991) Determinisme de la reproduction sexuee de *Phaesphaeria (leptosphaeria) nodorum*, agent de la septoriose du ble. I, heterothallisme et role des microspores. *Canadian journal of botany* **69**: 95-99.

Halket, J.M., Waterman, D., Przyborowska, A.M., Patel, R.K.P., Fraser, P.D., and Bramley, P.M. (2005) Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and LC/MS/MS. *Journal of Experimental Botany* **56**: 219-243.

Hamzehzarghani, H., Kushalappa, A.C., Dion, Y., Rioux, S., Comeau, A., Yaylayan, V., Marshall, W.D., and Mather, D.E. (2005) Metabolic profiling and factor analysis

to discriminate quantitative resistance in wheat cultivars against fusarium head blight. *Physiological and Molecular Plant Pathology* **66**: 119-133.

Hann, C.A., and Griffiths, E. (1976) Change in virulence of *Septoria nodorum* and *S. tritici* after passage through alternative hosts. *Transactions of the British Mycological Society* **66**: 337-340.

Harris, M.A., Clark, J., Ireland, A., Lomax, J., Ashburner, M., Foulger, R., Eilbeck, K., Lewis, S., Marshall, B., Mungall, C., Richter, J., Rubin, G.M., Blake, J.A., Bult, C., Dolan, M., Drabkin, H., Eppig, J.T., Hill, D.P., Ni, L., Ringwald, M., Balakrishnan, R., Cherry, J.M., Christie, K.R., Costanzo, M.C., Dwight, S.S., Engel, S., Fisk, D.G., Hirschman, J.E., Hong, E.L., Nash, R.S., Sethuraman, A., Theesfeld, C.L., Botstein, D., Dolinski, K., Feierbach, B., Berardini, T., Mundodi, S., Rhee, S.Y., Apweiler, R., Barrell, D., Camon, E., Dimmer, E., Lee, V., Chisholm, R., Gaudet, P., Kibbe, W., Kishore, R., Schwarz, E.M., Sternberg, P., Gwinn, M., Hannick, L., Wortman, J., Berriman, M., Wood, V., de la Cruz, N., Tonellato, P., Jaiswal, P., Seigfried, T., and White, R. (2004) The Gene Ontology (GO) database and informatics resource. *Nucleic Acids Res* **32**: D258-261.

Hazell, B.W., Nevalainen, H., and Attfield, P.V. (1995) Evidence that the *Saccharomyces cerevisiae* CIF1 (GGS1/TPS1) gene modulates heat shock response positively. *FEBS Letters* **377**: 457-460.

Hedjaroude, G.A. (1969) Etudes taxonomiques sur les Phaeosphaeria Miyake et leurs formes voisines (Ascomycetes). *Sydowia* **22**: 57-107.

Hewitt, H.G. (1998) *Fungicides in crop protection.* New York: CAB international.

Hicks, J.K., Yu, J.H., Keller, N.P., and Adams, T.H. (1997) *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA G alpha protein-dependent signaling pathway. *Embo J* **16**: 4916-4923.

Hirai, M.Y., Yano, M., Goodenowe, D.B., Kanaya, S., Kimura, T., Awazuhara, M., Arita, M., Fujiwara, T., and Saito, K. (2004) Integration of transcriptomics and metabolomics for understanding of global responses to nutritional stresses in Arabidopsis thaliana. *Proc Natl Acad Sci U S A* **101**: 10205-10210.

Horwitz, B.A., Sharon, A., Lu, S.W., Ritter, V., Sandrock, T.M., Yoder, O.C., and Turgeon, B.G. (1999) A G protein alpha subunit from *Cochliobolus heterostrophus* involved in mating and appressorium formation. *Fungal Genet Biol* **26**: 19-32.

Hottiger, T., De Virgilio, C., Hall, M.N., Boller, T., and Wiemken, A. (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast. II. Physiological concentrations of trehalose increase the thermal stability of proteins *in vitro*. *Eur J Biochem* **219**: 187-193.

Howard, K., Foster, S.G., Cooley, R.N., and Caten, C.E. (1999) Disruption, replacement, and cosuppression of nitrate assimilation genes in *Stagonospora nodorum*. *Fungal Genet Biol* **26**: 152-162.

Hsiang, T., and Goodwin, P.H. (2003) Distinguishing plant and fungal sequences in ESTs from infected plant tissues. *J Microbiol Methods* **54**: 339-351.

Ideker, T., Thorsson, V., Ranish, J.A., Christmas, R., Buhler, J., Eng, J.K., Bumgarner, R., Goodlett, D.R., Aebersold, R., and Hood, L. (2001) Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. *Science* **292**: 929-934.

Ishii, H., Fraaije, B.A., Sugiyama, T., Noguchi, K., Nishimura, K., Takeda, T., Amano, T., and Hollomon, D.W. (2001) Occurrence and molecular characterization of

strobilurin resistance in cucumber powdery mildew and downy mildew. *Phytopathology* **91**: 1166-1171.
Ivey, F.D., Hodge, P.N., Turner, G.E., and Borkovich, K.A. (1996) The G alpha i homologue gna-1 controls multiple differentiation pathways in *Neurospora crassa*. *Mol Biol Cell* **7**: 1283-1297.
Iwasa, T., Higashide, E., Yamamoto, H., and Shibata, M. (1971) Studies on validamycins, new antibiotics. II. Production and biological properties of validamycins A and B. *J Antibiot (Tokyo)* **24**: 107-113.
Jakupovic, M., Heintz, M., Reichmann, P., Mendgen, K., and Hahn, M. (2006) Microarray analysis of expressed sequence tags from haustoria of the rust fungus *Uromyces fabae*. *Fungal Genet Biol* **43**: 8-19.
Jennings, D. (1984) Polyol metabolism in fungi. *Adv.Microbial Physiol.* **25**: 149-193.
Jennings, D.B., Ehrenshaft, M., Pharr, D.M., and Williamson, J.D. (1998) Roles for mannitol and mannitol dehydrogenase in active oxygen-mediated plant defense. *PNAS* **95**: 15129-15133.
Jennings, D.B., Daub, M.E., Pharr, D.M., and Williamson, J.D. (2002) Constitutive expression of a celery mannitol dehydrogenase in tobacco enhances resistance to the mannitol-secreting fungal pathogen *Alternaria alternata*. *Plant J.* **32**: 41-49.
Junker, B.H., Klukas, C., and Schreiber, F. (2006) VANTED: a system for advanced data analysis and visualization in the context of biological networks. *BMC Bioinformatics* **7**: 109.
Kaasen, I., McDougall, J., and Strom, A.R. (1994) Analysis of the otsBA operon for osmoregulatory trehalose synthesis in *Escherichia coli* and homology of the OtsA and OtsB proteins to the yeast trehalose-6-phosphate synthase/phosphatase complex. *Gene* **145**: 9-15.
Kanehisa, M., and Goto, S. (2000) KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* **28**: 27-30.
Katona, Z.F., Sass, P., and Molnar-Perl, I. (1999) Simultaneous determination of sugars, sugar alcohols, acids and amino acids in apricots by gas chromatography-mass spectrometry. *Journal of Chromatography, A.* **847**: 91-102.
Keon, J., Antoniw, J., Rudd, J., Skinner, W., Hargreaves, J., and Hammond-Kosack, K. (2005) Analysis of expressed sequence tags from the wheat leaf blotch pathogen *Mycosphaerella graminicola* (anamorph *Septoria tritici*). *Fungal Genet Biol* **42**: 376-389.
Keun, H.C., Beckonert, O., Griffin, J.L., Richter, C., Moskau, D., Lindon, J.C., and Nicholson, J.K. (2002) Cryogenic Probe 13C NMR Spectroscopy of Urine for Metabonomic Studies. *Anal. Chem.* **74**: 4588-4593.
Kim, S., Ahn, I.P., and Lee, Y.H. (2001) Analysis of genes expressed during rice-*Magnaporthe grisea* interactions. *Mol Plant Microbe Interact* **14**: 1340-1346.
Klukas, C., Junker, B.H., and Schreiber, F. (2006) The VANTED software system for transcriptomics, proteomics and metabolomics analysis. *J. Pestic. Sci.* **31**: 289-292.
Kopka, J., Schauer, N., Krueger, S., Birkemeyer, C., Usadel, B., Bergmuller, E., Dormann, P., Weckwerth, W., Gibon, Y., Stitt, M., Willmitzer, L., Fernie, A.R., and Steinhauser, D. (2005) GMD@CSB.DB: the Golm Metabolome Database. *Bioinformatics* **21**: 1635-1638.
Kruger, J., Loubradou, G., Regenfelder, E., Hartmann, A., and Kahmann, R. (1998) Crosstalk between cAMP and pheromone signalling pathways in *Ustilago maydis*. *Mol Gen Genet* **260**: 193-198.
Lavoy, I.C. (2004) Wheat. In *World Book 2004*. Vol. 2006: World Book Inc.

Lee, B.N., and Adams, T.H. (1996) FluG and flbA function interdependently to initiate conidiophore development in *Aspergillus nidulans* through brlA beta activation. *Embo J* **15**: 299-309.
Lee, N., D'Souza, C.A., and Kronstad, J.W. (2003) Of smuts, blasts, mildews, and blights: cAMP signaling in phytopathogenic fungi. *Annual Review of Phytopathology* **41**: 399-427.
Leloir, L.F., and Cabib, E. (1953) The enzymic synthesis of trehalose phosphate. *Journal of the american chemical society* **75**: 5445-5446.
Lewis, D., and Smith, D. (1967) Sugar alcohols in fungi and green plants. I. DISTRIBUTION, PHYSIOLOGY AND METABOLISM. *New Phytologist.* **66**: 143-184.
Lingappa, B.T., and Sussman, A.S. (1959) Endogenous substrates of dormant, activated and germinating ascospores of *Neurospora tetrasperma*. *Plant Physiol* **34**: 466-472.
Link, T., Lohaus, G., Heiser, I., Mendgen, K., Hahn, M., and Voegele, R.T. (2005) Characterization of a novel NADP(+)-dependent D-arabitol dehydrogenase from the plant pathogen *Uromyces fabae*. *Biochem J* **389**: 289-295.
Liu, S., and Dean, R.A. (1997) G protein alpha subunit genes control growth, development, and pathogenicity of *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions* **10**: 1075-1086.
Liu, Z.H., Faris, J.D., Meinhardt, S.W., Ali, S., Rasmussen, J.B., and Friesen, T.L. (2004) Genetic and physical mapping of a gene conditioning sensitivity in wheat to a partially purified host-selective toxin produced by *Stagonospora nodorum*. . *Phytopathology* **94**: 1056-1060.
Makihara, F., Tsuzuki, M., Sato, K., Masuda, S., Nagashima, K.V., Abo, M., and Okubo, A. (2005) Role of trehalose synthesis pathways in salt tolerance mechanism of *Rhodobacter sphaeroides f. sp. denitrificans* IL106. *Arch Microbiol* **184**: 56-65.
Managbanag, J.R., and Torzilli, A.P. (2002) An analysis of trehalose, glycerol, and mannitol accumulation during heat and salt stress in a salt marsh isolate of *Aureobasidium pullulans*. *Mycologia* **94**: 384-391.
Marchler-Bauer, A., Panchenko, A.R., Shoemaker, B.A., Thiessen, P.A., Geer, L.Y., and Bryant, S.H. (2002) CDD: a database of conserved domain alignments with links to domain three-dimensional structure. *Nucl. Acids Res.* **30**: 281-283.
Marchler-Bauer, A., Anderson, J.B., Cherukuri, P.F., DeWeese-Scott, C., Geer, L.Y., Gwadz, M., He, S., Hurwitz, D.I., Jackson, J.D., Ke, Z., Lanczycki, C.J., Liebert, C.A., Liu, C., Lu, F., Marchler, G.H., Mullokandov, M., Shoemaker, B.A., Simonyan, V., Song, J.S., Thiessen, P.A., Yamashita, R.A., Yin, J.J., Zhang, D., and Bryant, S.H. (2005) CDD: a Conserved Domain Database for protein classification. *Nucl. Acids Res.* **33**: D192-196.
Mitchell, A.P. (1998) Dimorphism and virulence in *Candida albicans*. *Curr Opin Microbiol* **1**: 687-692.
Morzfeld, J., Koopmann, B., and Hoppe, H.H. (2004) Development of fungicide resistance in wheat and barley pathogens against strobilurins: a methodological approach. *Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz* **111**: 105-114.
Muller, J., Wiemken, A., and Aeschbacher, R. (1999) Trehalose metabolism in sugar sensing and plant development. *Plant Science Limerick* **147**: 37-47.
Myers, E.W., and Miller, W. (1988) Optimal alignments in linear space. *Comput. Appl. Biosci.* **4**: 11-17.

Narasimhan, K., Basheer, C., Bajic, V.B., and Swarup, S. (2003) Enhancement of plant-microbe interactions using a rhizosphere metabolomics-driven approach and its application in the removal of polychlorinated biphenyls. *Plant Physiol* **132**: 146-153.

Nicholson, J.K., Buckingham, M.J., and Sadler, P.J. (1983) High resolution 1H n.m.r. studies of vertebrate blood and plasma. *Biochemical Journal* **211**: 605-615.

Nicholson, J.K., Lindon, J.C., and Holmes, E. (1999) 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **29**: 1181-1189.

Nidetzky, B., and Eis, C. (2001) Alpha-retaining glucosyl transfer catalysed by trehalose phosphorylase from *Schizophyllum commune*: mechanistic evidence obtained from steady-state kinetic studies with substrate analogues and inhibitors. *Biochem J* **360**: 727-736.

Noubhani, A., Bunoust, O., Rigoulet, M., and Thevelein, J.M. (2000) Reconstitution of ethanolic fermentation in permeabilized spheroplasts of wild-type and trehalose-6-phosphate synthase mutants of the yeast *Saccharomyces cerevisiae*. *Eur J Biochem* **267**: 4566-4576.

O'Brien, T.P., and McCully, M.E. (1981) *The study of plant structure: Principles and selected methods.* Melbourne: Termarcarphi Pty. Ltd.

O'Neil, M.J. (2001) *The Merck index : an encyclopedia of chemicals, drugs, and biologicals* Whitehouse Station, NJ: Merck.

O'Reilly, P., and Downes, M.J. (1986) Form of survival of Septoria nodorum on symptomless winter wheat. *Transactions of the British Mycological Society* **86**: 381-385.

Oliver, R.P., and Ipcho, S.V.S. (2004) *Arabidopsis* pathology breathes new life into the necrotrophs vs. biotrophs classification of fungal pathogens. *Molecular Plant Pathology* **5**: 347-352.

Oliver, S.G., Winson, M.K., Kell, D.B., and Baganz, F. (1998) Systematic functional analysis of the yeast genome. *Trends Biotechnol* **16**: 373-378.

Oreskes, N. (2004) BEYOND THE IVORY TOWER: The Scientific Consensus on Climate Change. *Science* **306**: 1686-.

Pail, M., Peterbauer, T., Seiboth, B., Hametner, C., Druzhinina, I., and Kubicek, C.P. (2004) The metabolic role and evolution of L-arabinitol 4-dehydrogenase of *Hypocrea jecorina*. *Eur J Biochem* **271**: 1864-1872.

Parsley, T.B., Segers, G.C., Nuss, D.L., and Dawe, A.L. (2003) Analysis of altered G-protein subunit accumulation in *Cryphonectria parasitica* reveals a third G alpha homologue. *Current Genetics* **43**: 24-33.

Pastushok, M., and Axelrod, D.E. (1976) Effect of glucose, ammonium and media maintenance on the time of conidiophore initiation by surface colonies of *Aspergillus nidulans*. *J Gen Microbiol* **94**: 221-224.

Pauling, L., Robinson, A.B., Teranishi, R., and Cary, P. (1971) Quantitative analysis of urine vapor and breath by gas-liquid partition chromatography. *Proc Natl Acad Sci U S A* **68**: 2374-2376.

Pearson, K. (1901) On lines and planes of closest fit to systems of points in space. *Philosophical Magazine* **2**: 559-572.

Petzold, E.W., Himmelreich, U., Mylonakis, E., Rude, T., Toffaletti, D., Cox, G.M., Miller, J.L., and Perfect, J.R. (2006) Characterization and regulation of the trehalose synthesis pathway and its importance in the pathogenicity of Cryptococcus neoformans. *Infect Immun* **74**: 5877-5887.

Pruzinska, A., Tanner, G., Aubry, S., Anders, I., Moser, S., Muller, T., Ongania, K.H., Krautler, B., Youn, J.Y., Liljegren, S.J., and Hortensteiner, S. (2005) Chlorophyll breakdown in senescent *Arabidopsis* leaves. Characterization of chlorophyll catabolites and of chlorophyll catabolic enzymes involved in the degreening reaction. *Plant Physiol* **139**: 52-63.

Purcell, E.M., Torrey, H.C., and Pound, R.V. (1946) Resonance Absorption by Nuclear Magnetic Moments in a Solid. *Physical Review* **69**: 37.

Ratnakumar, S., and Tunnacliffe, A. (2006) Intracellular trehalose is neither necessary nor sufficient for desiccation tolerance in yeast. *FEMS Yeast Res* **6**: 902-913.

Reithner, B., Brunner, K., Schuhmacher, R., Peissl, I., Seidl, V., Krska, R., and Zeilinger, S. (2005) The G protein alpha subunit Tga1 of *Trichoderma atroviride* is involved in chitinase formation and differential production of antifungal metabolites. *Fungal Genet Biol* **42**: 749-760.

Richard, P., Londesborough, J., Putkonen, M., Kalkkinen, N., and Penttila, M. (2001) Cloning and expression of a fungal L-arabinitol 4-dehydrogenase gene. *J Biol Chem* **276**: 40631-40637.

Robertson, D.G., Reily, M.D., Albassam, M., and Dethloff, L.A. (2001) Metabonomic assessment of vasculitis in rats. *Cardiovasc Toxicol* **1**: 7-19.

Roessner, U., Wagner, C., Kopka, J., Trethewey, R.N., and Willmitzer, L. (2000) Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. *Plant Journal* **23**: 131-142.

Roessner, U., Patterson, J.H., Forbes, M.G., Fincher, G.B., Langridge, P., and Bacic, A. (2006) An Investigation of Boron Toxicity in Barley using Metabolomics. *Plant Physiol*.

Ruijter, G.J., Bax, M., Patel, H., Flitter, S.J., van de Vondervoort, P.J., de Vries, R.P., vanKuyk, P.A., and Visser, J. (2003) Mannitol is required for stress tolerance in *Aspergillus niger* conidiospores. *Eukaryot Cell* **2**: 690-698.

Sacadura, N.T., and Saville, B.J. (2003) Gene expression and EST analyses of *Ustilago maydis* germinating teliospores. *Fungal Genet Biol* **40**: 47-64.

Sampedro, J.G., and Uribe, S. (2004) Trehalose-enzyme interactions result in structure stabilization and activity inhibition. The role of viscosity. *Mol Cell Biochem* **256-257**: 319-327.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**: 5463-5467.

Sato, S., Soga, T., Nishioka, T., and Tomita, M. (2004) Simultaneous determination of the main metabolites in rice leaves using capillary electrophoresis mass spectrometry and capillary electrophoresis diode array detection. *Plant J* **40**: 151-163.

Schauer, Steinhauser, Strelkov, Schomburg, Allison, Moritz, Lundgren, Roessner, T., Forbes, Willmitzer, Fernie, and Kopka (2005) GC-MS libraries for the rapid identification of metabolites in complex biological samples. *FEBS Letters* **579**: 1332-1337.

Schena, M., Shalon, D., Davis, R.W., and Brown, P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**: 467-470.

Seitz, L., Sauer, D., Burroughs, R., Mohr, H., and Hubbard, J. (1979) Ergosterol as a measure of fungal growth. *Phytopath.* **69**: 1202-1203.

Semova, N., Storms, R., John, T., Gaudet, P., Ulycznyj, P., Min, X.J., Sun, J., Butler, G., and Tsang, A. (2006) Generation, annotation, and analysis of an extensive *Aspergillus niger* EST collection. *BMC Microbiol* **6**: 7.

Seo, J.A., and Yu, J.H. (2006) The phosducin-like protein PhnA is required for Gbetagamma-mediated signaling for vegetative growth, developmental control, and toxin biosynthesis in *Aspergillus nidulans*. *Eukaryot Cell* **5**: 400-410.

Sexton, A.C., Cozijnsen, A.J., Keniry, A., Jewell, E., Love, C.G., Batley, J., Edwards, D., and Howlett, B.J. (2006) Comparison of transcription of multiple genes at three developmental stages of the plant pathogen *Sclerotinia sclerotiorum*. *FEMS Microbiol Lett* **258**: 150-160.

Shah, D., Bergstrom, G.C., and Ueng, P.P. (1995) Initiation of Septoria nodorum blotch epidemics in winter wheat by seedborne *Stagonospora nodorum*. *Phytopathology,* **85**: 452-457.

Shah, D.A., and Bergstrom, G.C. (2000) Temperature dependent seed transmission of *Stagonospora nodorum* in wheat. *European journal of plant pathology*: 837-842.

Shah, D.A., Bergstrom, G.C., and Ueng, P.P. (2001) Foci of *Stagonospora nodorum* blotch in winter wheat before canopy development. *Phytopathology* **91**: 642-647.

Shah, D.A., and Bergstrom, G.C. (2002) A rainfall-based model for predicting the regional incidence of wheat seed infection by *Stagonospora nodorum* in New York. *Phytopathology* **92**: 511-518.

Shimizu, K., and Keller, N.P. (2001) Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. *Genetics* **157**: 591-600.

Shimizu, K., Hicks, J.K., Huang, T.P., and Keller, N.P. (2003) Pka, Ras and RGS protein interactions regulate activity of AflR, a Zn(II)2Cys6 transcription factor in Aspergillus nidulans. *Genetics* **165**: 1095-1104.

Shipton, P.J., Boyd, W.R.J., Rosielle, A.A., and Shearer, B.L. (1971) The common Septoria diseases of wheat. *Botanical Review* **37**: 231-262.

Singer, M.A., and Lindquist, S. (1998a) Multiple effects of trehalose on protein folding *in vitro* and *in vivo*. *Molecular Cell* **1**: 639-648.

Singer, M.A., and Lindquist, S. (1998b) Thermotolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose. *Trends in Biotechnology* **16**: 460-468.

Soanes, D.M., Skinner, W., Keon, J., Hargreaves, J., and Talbot, N.J. (2002) Genomics of phytopathogenic fungi and the development of bioinformatic resources. *Mol Plant Microbe Interact* **15**: 421-427.

Soanes, D.M., and Talbot, N.J. (2005) A bioinformatic tool for analysis of EST transcript abundance during infection-related development by *Magnaporthe grisea*. *Molecular Plant Pathology* **6**: 503-512.

Soanes, D.M., and Talbot, N.J. (2006) Comparative genomic analysis of phytopathogenic fungi using expressed sequence tag (EST) collections. *Molecular Plant Pathology* **7**: 61-70.

Soga, T., Ohashi, Y., Ueno, Y., Naraoka, H., Tomita, M., and Nishioka, T. (2003) Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. *J Proteome Res* **2**: 488-494.

Solomon, P.S., Thomas, S.W., Spanu, P., and Oliver, R.P. (2003) The utilisation of di/tripeptides by *Stagonospora nodorum* is dispensable for wheat infection. *Physiological and Molecular Plant Pathology* **63**: 191-199.

Solomon, P.S., Lee, R.C., Wilson, T.J.G., and Oliver, R.P. (2004a) Pathogenicity of *Stagonospora nodorum* requires malate synthase. *Molecular Microbiology* **53**: 1065-1073.

Solomon, P.S., and Oliver, R.P. (2004) Functional characterisation of glyoxalase I from the fungal wheat pathogen *Stagonospora nodorum*. *Current Genetics* **46**: 115-121.
Solomon, P.S., Tan, K., Sanchez, P., Cooper, R.M., and Oliver, R.P. (2004b) The disruption of a G alpha subunit sheds new light on the pathogenicity of *Stagonospora nodorum* on wheat. *Molecular Plant Microbe Interactions* **17**: 456-466.
Solomon, P.S., Tan, K., and Oliver, R.P. (2005a) Mannitol 1-phosphate metabolism is required for sporulation in planta of the wheat pathogen *Stagonospora nodorum*. *Molecular Plant Microbe Interactions* **18**: 110-115.
Solomon, P.S., Waters, O.D., Simmonds, J., Cooper, R.M., and Oliver, R.P. (2005b) The Mak2 MAP kinase signal transduction pathway is required for pathogenicity in *Stagonospora nodorum*. *Curr Genet* **48**: 60-68.
Solomon, P.S., Jorgens, C.I., and Oliver, R.P. (2006a) Delta-aminolaevulinic acid synthesis is required for virulence of the wheat pathogen *Stagonospora nodorum*. *Microbiology* **152**: 1533-1538.
Solomon, P.S., Lowe, R.G., Trengove, R.D., Rechberger, J., and Oliver, R.P. (2006b) Normalization of metabolites in heterogenous systems using genomics. *Anal Biochem* **350**: 156-158.
Solomon, P.S., Lowe, R.G.T., Tan, K.-C., Waters, O.D.C., and Oliver, R.P. (2006c) *Stagonospora nodorum:* cause of stagonospora nodorum blotch of wheat. *Molecular Plant Pathology* **7**: 147-156.
Solomon, P.S., Rybak, K., Trengove, R.D., and Oliver, R.P. (2006d) Investigating the role of calcium/calmodulin-dependent protein kinases in *Stagonospora nodorum*. *Mol Microbiol* **62**: 367-381.
Solomon, P.S., Waters, O.D., Jorgens, C.I., Lowe, R.G., Rechberger, J., Trengove, R.D., and Oliver, R.P. (2006e) Mannitol is required for asexual sporulation in the wheat pathogen *Stagonospora nodorum* (glume blotch). *Biochem J* **399**: 231-239.
Solomon, P.S., Wilson, T.J.G., Rybak, K., Parker, K., Lowe, R.G.T., and Oliver, R.P. (2006f) Structural characterisation of the interaction between *Triticum aestivum* and the dothideomycete pathogen *Stagonospora nodorum*. *European Journal of Plant Pathology* **114**: 275-282.
Statistics, T.A.B.o. (2006) The Australian Wheat Industry. In *Year Book Australia, 2006 (1301.0)*. Vol. 2006: Australian Bureau of Statistics.
Stein, S.E. (1999) An integrated method for spectrum extraction and compound identification from GC/MS data. *Journal of the American Society of Mass Spectrometry* **10**: 770-781.
Stoop, J.M.H., and Mooibroek, H. (1998) Cloning and Characterization of NADP-Mannitol Dehydrogenase cDNA from the Button Mushroom, *Agaricus bisporus*, and Its Expression in Response to NaCl Stress. *Appl. Environ. Microbiol.* **64**: 4689-4696.
Stoughton, R.B. (2005) Applications of DNA microarrays in biology. *Annu Rev Biochem* **74**: 53-82.
Stover, R.W., Francl, L.J., and Jordahl, J.G. (1996) Tillage and fungicide management of foliar diseases in a spring wheat monoculture. *J. Prod. Agric.* **9**: 261-265.
Stryer, L. (1996) *Biochemistry.* New York: W. H. Freeman and company.
Szabo, L.J., Small, G.M., and Lazarow, P.B. (1989) The nucleotide sequence of POX18, a gene encoding a small oleate-inducible peroxisomal protein from *Candida tropicalis*. *Gene* **75**: 119-126.

The_Broad_Institute (2005) *Stagonospora nodorum* Sequencing Project. Broad Institute of Harvard and MIT.

Thevelein, J.M. (1984) Regulation of trehalose mobilization in fungi. *Microbiol Rev* **48**: 42-59.

Thevelein, J.M., and Hohmann, S. (1995) Trehalose synthase: guard to the gate of glycolysis in yeast? *Trends Biochem Sci* **20**: 3-10.

Thines, E., Weber, R.W.S., and Talbot, N.J. (2000) MAP kinase and protein kinase A-dependent mobilization of triacylglycerol and glycogen during appressorium turgor generation by *Magnaporthe grisea*. *Plant Cell* **12**: 1703-1718.

Tohge, T., Nishiyama, Y., Hirai, M.Y., Yano, M., Nakajima, J.-i., Awazuhara, M., Inoue, E., Takahashi, H., Goodenowe, D.B., Kitayama, M., Noji, M., Yamazaki, M., and Saito, K. (2005) Functional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor. *The Plant Journal* **42**: 218-235.

Trail, F., and Xu, H.X. (2002) Purification and characterization of mannitol dehydrogenase and identification of the corresponding cDNA from the head blight fungus, *Gibberella zeae* (*Fusarium graminearum*). *Phytochemistry* **61**: 791-796.

Trail, F., Xu, J.R., San Miguel, P., Halgren, R.G., and Kistler, H.C. (2003) Analysis of expressed sequence tags from *Gibberella zeae* (anamorph *Fusarium graminearum*). *Fungal Genet Biol* **38**: 187-197.

Tsitsigiannis, D.I., and Keller, N.P. (2006) Oxylipins act as determinants of natural product biosynthesis and seed colonization in *Aspergillus nidulans. Mol Microbiol* **59**: 882-892.

Urbanczyk-Wochniak, E., Luedemann, A., Kopka, J., Selbig, J., Roessner-Tunali, U., Willmitzer, L., and Fernie, A.R. (2003) Parallel analysis of transcript and metabolic profiles: a new approach in systems biology. *EMBO Rep* **4**: 989-993.

Van Aelst, L., Hohmann, S., Bulaya, B., de Koning, W., Sierkstra, L., Neves, M.J., Luyten, K., Alijo, R., Ramos, J., and Coccetti, P. (1993) Molecular cloning of a gene involved in glucose sensing in the yeast *Saccharomyces cerevisiae*. *Mol Microbiol* **8**: 927-943.

Velculescu, V.E., Zhang, L., Vogelstein, B., and Kinzler, K.W. (1995) Serial analysis of gene expression. *Science* **270**: 484-487.

Villas-Boas, S.G., Moxley, J.F., Akesson, M., Stephanopoulos, G., and Nielsen, J. (2005) High-throughput metabolic state analysis: the missing link in integrated functional genomics of yeasts. *Biochem J* **388**: 669-677.

Voegele, R.T., Hahn, M., Lohaus, G., Link, T., Heiser, I., and Mendgen, K. (2005) Possible roles for mannitol and mannitol dehydrogenase in the biotrophic plant pathogen *Uromyces fabae*. *Plant Physiology* **137**: 190-198.

Wang, Z.Y., Thornton, C.R., Kershaw, M.J., Li, D.B., and Talbot, N.J. (2003) The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus Magnaporthe grisea. *Molecular Microbiology* **47**: 1601-1612.

Ward, J.H., Jr. (1963) Hierarchical grouping to optimize an objective function. *Journal of the American Statistical Association* **58**: 236-244.

Welthagen, W., Shellie, R., Spranger, J., Ristow, M., Zimmermann, R., and Fiehn, O. (2005) Comprehensive two-dimensional gas chromatography, time-of-flight mass spectrometry (GC x GC-TOF) for high resolution metabolomics: biomarker discovery on spleen tissue extracts of obese NZO compared to lean C57BL/6 mice. *Metabolomics* **1**: 65-73.

Wiggers, H.A.L. (1832). *Ann. Pharmacol.* **1**: 129-182.

Wilbur, W.J., and Lipman, D.J. (1983) Rapid similarity searches of nucleic acid and protein data banks. *PNAS* **80**: 726-730.

Wilkinson, H.H., Ramaswamy, A., Sim, S.C., and Keller, N.P. (2004) Increased conidiation associated with progression along the sterigmatocystin biosynthetic pathway. *Mycologia* **96**: 1190-1198.

Wilson, R.A., and Talbot, N.J. (2006) The trehalose 6 phosphate synthase gene of *Magnaporthe grisea* plays an intergrative role in regulation of carbon and nitrogen metabolism and is essential for plant pathogenicity. In *8th European conference on fungal genetics*. Druzhinina, I.S., Kopchinskiy, A.G. and Kubicek, C.P. (eds.) Vienna, Austria, pp. 192.

Witteveen, C.F.B., and Visser, J. (1995) Polyol pools in *Aspergillus niger*. *FEMS Microbiol Letters.* **134**: 57-62.

Wolf, J.C., and Mirocha, C.J. (1977) Control of sexual reproduction in *Gibberella zeae (Fusarium roseum "Graminearum")*. *Applied and Environmental Microbiology* **33**: 546-550.

Wrabl, J.O., and Grishin, N.V. (2001) Homology between O-linked GlcNAc transferases and proteins of the glycogen phosphorylase superfamily. *J Mol Biol* **314**: 365-374.

Yager, L.N., Kurtz, M.B., and Champe, S.P. (1982) Temperature-shift analysis of conidial development in *Aspergillus nidulans*. *Dev Biol* **93**: 92-103.

Zaragoza, O., Blazquez, M.A., and Gancedo, C. (1998) Disruption of the *Candida albicans Tps1* gene encoding trehalose-6-phosphate synthase impairs formation of hyphae and decreases infectivity. *J Bacteriol* **180**: 3809-3815.

Zuber, S., Hynes, M.J., and Andrianopoulos, A. (2003) The G-protein alpha-subunit GasC plays a major role in germination in the dimorphic fungus *Penicillium marneffei*. *Genetics* **164**: 487-499.

Zweytick, D., Athenstaedt, K., and Daum, G. (2000) Intracellular lipid particles of eukaryotic cells. *Biochimica et Biophysica Acta* **1469**: 101-120.

Chapter 10

Appendices

10.1 Plasmid and construct maps

bluescript and ptriplex2

pSport1 and pAN8-1

pBSKphleo and pGEMt easy

pGEM t easy tps 3’ and pBSK phleo tps3’

pTPSKO and abd1 construct

10.2 Metabolomics retention-time libraries

10.2.1 The MPIMP "Pol_fa TMS" library.

Courtesy of the Max Plank Institute of Molecular Plant Physiology, Golm, Germany.

Observed RT (minutes)	Predicted RT for non-RT-locked data (minutes)	Peak Identity
4.4930	6.2623	PROPAN-1,2-DIOL,O,O-TMS
4.5120	6.2816	ETHYLENEGLYCOL,O,O-TMS
6.4470	8.2451	LACTIC ACID,O,O-TMS
6.6850	8.4866	GLYOXYLIC ACID MEOX TMS
6.6850	8.4866	GLYOXYLIC ACID MEOX TMS
6.6850	8.4866	GLYOXYLIC ACID MEOX TMS
7.0620	8.8691	HEXANOIC ACID,O-TMS
7.2000	9.0091	GLYCOLIC ACID,O,O-TMS
7.2060	9.0152	ALANINE,N,O-TMS
8.0390	9.8605	GLYCINE,N,O-TMS
8.4340	10.2613	PYRUVIC ACID MEOX TMS
8.9270	10.7615	NORVALINE,O-TMS
8.9550	10.7899	HYDROXYBUTANOIC ACID,O,O-TMS
9.5080	11.3511	C7 TMS
9.6290	11.4738	2-OXOISOVALERIC ACID MEOX1 TMS
9.7920	11.6392	2-OXOBUTYRIC ACID MEOX1 TMS
9.8690	11.7174	VALINE,N,O-TMS
10.0610	11.9122	3-METHYLAMINOL-1,2-PROPANDIOL,O,O-TMS
10.1200	11.9721	ETHANOLAMINE,N,N,O-TMS
10.1450	11.9974	OXALIC ACID TMS
10.3650	12.2207	3 HO-6:0 ME TMS
10.5250	12.3830	2-OXOISOVALERIC ACID MEOX2 TMS
10.5270	12.3850	NORVALINE,N,O-TMS
10.5750	12.4338	2-OXOVALERIC ACID MEOX1 TMS
10.6330	12.4926	GLYCEROL 3TMS
10.6850	12.5454	3-HYDROXYPYRIDINE,O-TMS
10.8990	12.7625	GLYCERALDEHYD MEOX1 2TMS
10.9540	12.8183	2-AMINO-2-METHYL-1,3-PROPANDIOL 3TMS
10.9670	12.8315	2-METHYLSERINE,N,O,O-TMS
10.9670	12.8315	2-METHYLSERINE,O,O-TMS
11.0580	12.9239	4-HYDROXYPYRIDINE,O-TMS
11.1170	12.9837	THREONINOL,O,O-TMS
11.1830	13.0507	NORLEUCINE,O-TMS
11.2030	13.0710	2-OXOISOCAPROIC ACID MEOX1 TMS
11.3150	13.1846	LEUCINE, N,O-TMS
11.4130	13.2841	GLYCERALDEHYD MEOX2 TMS
11.6580	13.5327	PHENETHYLAMINE,N-TMS
11.7150	13.5905	2-OXOVALERIC ACID MEOX2 TMS
11.9250	13.8036	ISOLEUCINE,N,O-TMS
12.1630	14.0451	GLYCINE,N,N,O-TMS
12.4950	14.3820	DIETHANOLAMINE,O,O-TMS
12.5400	14.4276	SERINE,O,O-TMS
12.5410	14.4287	2-OXOISOCAPROIC ACID MEOX2 TMS
12.7800	14.6712	NORLEUCINE,N,O-TMS
12.9300	14.8234	3-METHYLAMINOL-1,2-PROPANDIOL,N,O,O-TMS
12.9460	14.8396	PROLINE,N,O-TMS
13.0590	14.9543	OXAMIC ACID,O-TMS MEOX
13.0620	14.9573	PHOSPHORIC ACID,O,O,O-TMS
13.3460	15.2455	GLYCERIC ACID,O,O,O-TMS
13.3990	15.2993	UREA 2TMS
13.6590	15.5631	BENCOIC ACID TMS
13.8240	15.7305	SERINE,N,O,O-TMS
14.0630	15.9730	FUMARIC ACID (2TMS)
14.1010	16.0116	PIPECOLIC ACID,O-TMS
14.2300	16.1425	THREONINE,N,O,O-TMS
14.2470	16.1597	ALLOTHREONINE,N,O,O-TMS

Observed RT (minutes)	Predicted RT for non-RT-locked data (minutes)	Peak Identity
14.2470	16.1597	3-HYDROXYPYRUVIC ACID MEOX2 TMS
14.4060	16.3211	SUCCINIC ACID 2TMS
14.4150	16.3302	C9 TMS
14.5010	16.4175	PIPECOLIC ACID,N,O-TMS
14.7490	16.6691	DIETHANOLAMINE,N,O,O-TMS
14.7950	16.7158	3 HO-8:0 ME TMS
15.1540	17.0801	3 HO-8:1 ME TMS
15.2010	17.1278	OXAMIC ACID,N,O-TMS MEOX1
15.2430	17.1704	B-ALANINE TMS
15.2470	17.1744	OXAMIDE,N,N-TMS
15.3150	17.2434	NICOTINIC ACID TMS
15.3200	17.2485	OXAMIC ACID,N,O-TMS MEOX2
15.7450	17.6798	ERYTHROSE MEOX1 3TMS
15.9860	17.9243	HOMOSERINE 3TMS
16.1000	18.0400	3-HYDROXYPYRUVIC ACID MEOX1 TMS
16.1290	18.0694	ERYTHRITOL TMS
16.2110	18.1526	NORVALINE,N,N,O-TMS
16.2680	18.2104	ERYTHROSE MEOX2 TMS
16.6170	18.5646	GLUTARIC ACID,O,O-TMS
16.7590	18.7087	OXAMIDE,N,N-TMS MEOX
17.0050	18.9583	2-METHYLMALIC ACID 3TMS
17.1150	19.0699	THYMINE 2TMS
17.1220	19.0770	S-METHYLCYSTEINE
17.1220	19.0770	S-METHYLCYSTEINE,N,O-TMS
17.1790	19.1348	UNDECANOIC ACID ME
17.8900	19.8563	MALIC ACID TMS
17.9490	19.9162	L-HYDROXYPROLINE,N,O,O-TMS
18.0510	20.0196	NORLEUCINE,N,N,O-TMS
18.5900	20.5666	ASPARTIC ACID,N,O,O-TMS
18.6320	20.6092	1,3-DIAMINOPROPANE,N,N,N,N-TMS
18.7400	20.7188	THREONIC ACID,O,O,O,O-TMS
19.0730	21.0567	C11 TMS
19.1700	21.1551	1,4-DIDEOXY-1,4-IMINO-D-ARABINITOL 3TMS
19.2420	21.2282	3 HO-10:0 ME TMS
19.2870	21.2738	1,4-DIDEOXY-1,4-IMINO-D-ARABINITOL 3TMS
19.3490	21.3367	METHIONINE,N,O-TMS
19.4350	21.4240	ASPARAGINE,N,N,N,O-TMS
19.4530	21.4423	DODECANOIC ACID ME
19.5600	21.5508	CYSTEINE,N,O,S-TMS
19.7250	21.7183	3 HO-10:1 ME TMS
19.7780	21.7720	L-ARGININE TMS
19.9840	21.9811	SALICYLIC ACID 2TMS
20.0730	22.0714	LYXOSE,O,O,O,O-TMS MEOX1
20.4640	22.4681	6-HYDROXYNICOTINIC ACID,O,O-TMS
20.5120	22.5168	RIBITOL TMS
20.5250	22.5300	XYLOSE MEOX1 4TMS
20.5310	22.5361	HOMOGLUTAMINE TMS
20.5700	22.5757	ARABINOSE MEOX2 4TMS
20.5700	22.5757	ARABINOSE MEOX2 4TMS
20.5930	22.5990	LYXOSE,O,O,O,O-TMS MEOX2
20.6680	22.6751	XYLITOL 5TMS
20.6860	22.6934	RIBOSE MEOX1 4TMS
20.7100	22.7177	GLUTAMIC ACID 3TMS
20.7350	22.7431	ARABINOSE MEOX1 4TMS
20.8230	22.8324	RIBOSE MEOX2 4TMS
20.8920	22.9024	XYLOSE MEOX2 4TMS
20.9950	23.0069	PYROGLUTAMIC ACID 2TMS
21.1460	23.1601	PUTRESCINE,N,N,N,N-TMS
21.2100	23.2251	RHAMNOSE MEOX2 4TMS
21.2360	23.2515	ALPHA-KETOGLUTARIC ACID MEOX2 2TMS
21.2430	23.2586	3-OXOGLUTARIC ACID MEOX2 TMS
21.2900	23.3063	LAURIC ACID TMS
21.3130	23.3296	XYLULOSE MEOX 4TMS
21.3570	23.3742	RHAMNOSE MEOX1 4TMS
21.4200	23.4382	TARTARIC ACID,O,O,O,O-TMS
21.7010	23.7233	FUCOSE MEOX1 4TMS
21.7070	23.7294	TRIDECANOIC ACID ME
21.7170	23.7395	CYTOSINE 2TMS

Observed RT (minutes)	Predicted RT for non-RT-locked data (minutes)	Peak Identity
21.7890	23.8126	HOMOCYSTEINE,N,N,O-TMS
21.7970	23.8207	GLUTAMINE,N,N,N,O-TMS
21.8570	23.8816	FUCOSE MEOX2 4TMS
21.9680	23.9942	4-HYDROXYBENZOIC ACID,O,O-TMS
21.9960	24.0226	METHYLCYTOSINE 2TMS
22.0830	24.1109	PHENYLALANINE,N,O-TMS
22.1420	24.1708	S-METHYLCYSTEINE,N,N,O-TMS
22.2420	24.2723	3-OXOGLUTARIC ACID MEOX1 TMS
22.2450	24.2753	ALPHA-KETOGLUTARIC ACID MEOX1 2TMS
22.4190	24.4519	1HO-14:0 TMS
22.6930	24.7299	CADAVERINE,N,N,N,N-TMS
23.0100	25.0515	2-AMINO-ADIPINIC ACID 3TMS
23.0390	25.0810	ASPARAGINE,N,N,O-TMS
23.3730	25.4199	C13 TMS
23.4060	25.4534	3 HO-12:0 ME TMS
23.4970	25.5457	ORNITHINE,N,N,N',O-TMS
23.5110	25.5599	GLYCEROL-2-PHOSPHATE,O,O,O,O-TMS
23.5620	25.6117	GLYCERO-3-PHOSPHATE 4TMS
23.8190	25.8724	TETRADECANOIC ACID ME
23.9770	26.0328	3 HO-12:2 ME TMS
24.1340	26.1921	CIS-9-TETRADECENOIC ACID ME
24.2990	26.3595	MANNITOL TMS
24.4130	26.4752	QUINIC ACID TMS
24.5300	26.5939	FRUCTOSE MEOX1 5TMS
24.5960	26.6609	ACONITIC ACID 3TMS
24.5990	26.6639	MANNOSE MEOX TMS
24.6290	26.6943	SORBTOL TMS
24.6880	26.7542	GALACTITOL TMS
24.8960	26.9653	FRUCTOSE MEOX2 5TMS
24.8990	26.9683	GALACTOSE MEOX1 TMS (Peak A)
24.9120	26.9815	SHIKIMIC ACID TMS
25.1320	27.2047	GLUCOSE MEOX1 5TMS (peak a)
25.1520	27.2250	PUTRESCINE + CO2 4TMS
25.2230	27.2971	CITRIC ACID TMS
25.2480	27.3224	GALACTOSE MEOX2 TMS (Peak B)
25.2650	27.3397	GLUCOSAMINE MEOX1 TMS (Peak A)
25.2910	27.3661	GLUTAMINE,N,N,O-TMS
25.3610	27.4371	GLUCOSE MEOX2 5TMS (peak B)
25.5450	27.6238	LYSINE,N,N,N',O-TMS
25.5610	27.6400	2-PHOSPHOGLYCERATE TMS
25.6140	27.6938	ISOCITRIC ACID TMS
25.7000	27.7811	ONONITOL TMS
25.8770	27.9607	PENTADECANOIC ACID ME
26.0290	28.1149	3-PHOSPHOGLYCERATE TMS
26.1900	28.2783	CIS-10-PENTADECENOIC ACID ME
26.2010	28.2895	GLUCOSAMINE MEOX2 TMS
26.3640	28.4549	1HO-16:0 TMS
26.4090	28.5005	ALLANTOIN,N,N,N,N,N-TMS
26.5300	28.6233	GLUCURONIC ACID MEOX1 5TMS peak A
26.5810	28.6750	TYRAMINE,N,O-TMS
26.6690	28.7643	GLUCONIC ACID,O,O,O,O,O,O-TMS
26.7230	28.8191	GLUCURONIC ACID MEOX2 5TMS Peak B
26.8950	28.9937	GALACTURONIC ACID MEOX1 5TMS Peak A
26.8980	28.9967	MANNONO-DELTA-LACTAM MEOX 4TMS
27.1610	29.2636	GALACTURONIC ACID MEOX2 5TMS Peak B
27.3160	29.4208	C15 TMS
27.4040	29.5101	3 HO-14:2 ME TMS
27.4040	29.5101	3 HO-14:3 ME TMS
27.4110	29.5172	SACCHARIC ACID TMS
27.7010	29.8115	INOSITOL,O,O,O,O,O,O-TMS
27.7320	29.8430	ASCORBIC ACID TMS
27.7410	29.8521	ALLANTOIN,N,N,N,N-TMS
27.8320	29.9444	HEXADECANOIC ACID ME
27.9180	30.0317	3 HO-14:4 ME TMS
27.9610	30.0753	CIS-9-HEXADECENOIC ACID ME
27.9930	30.1078	D-MANNONO-DELTA-LACTAM 4TMS
28.1560	30.2732	4-AMINOBUTYRIC ACID 3TMS
28.1560	30.2732	GLUCURONIC ACID-E-LACTONE MEOX TMS

Observed RT (minutes)	Predicted RT for non-RT-locked data (minutes)	Peak Identity
28.2030	30.3209	GLUCARIC ACID 1,4LACTONE MEOX1 TMS
28.5320	30.6547	GLUCOHEPTULOSE MEOX1 TMS
28.6880	30.8130	GLUCARIC ACID 1,4LACTONE MEOX2 TMS
28.8710	30.9987	GLUCOHEPTULOSE MEOX2 TMS
29.3610	31.4959	DOPAMINE,N,N,O,O-TMS
29.7100	31.8500	HEPTADECANOIC ACID ME
29.8390	31.9809	CIS-10-HEPTADECENOIC ACID ME
29.9970	32.1413	1HO-18:0 TMS
30.2780	32.4264	NORADRENALINE,N,N,O,O,O-TMS
30.2850	32.4335	SPERMIDINE,N,N,N,N,N-TMS
30.3980	32.5482	NORMETHYLADRENALINE 4TMS
30.6150	32.7683	GLUCOHEPTONIC ACID TMS
30.6150	32.7683	GLUCOHEPTONIC ACID TMS
30.7460	32.9013	3HO-16:1 ME TMS
30.7640	32.9195	3HO-16:0 ME TMS
30.9270	33.0849	RIBOSE-5-PHOSPHATE TMS
31.0030	33.1620	3HO-16:2 ME TMS
31.4980	33.6643	3HO-16:3 ME TMS
31.5120	33.6785	OCTADECANOIC ACID ME
31.5210	33.6877	CIS-9-OCTADECENOIC ACID ME
31.6130	33.7810	TRANS-9-OCTADECENOIC ACID ME
31.7260	33.8957	INDOLE-3-ACETIC ACID 2TMS
31.8520	34.0235	CIS-9,12-OCTADECADIENOIC ACID ME
31.9440	34.1169	CIS-6,9,12-OCTADECATRIENOIC ACID ME
32.1000	34.2752	TRANS-9,12-OCTADECADIENOIC ACID ME
32.4030	34.5826	CIS-9,12,15-OCTADECATIENOIC ACID ME
32.6240	34.8069	CYSTATHIONINE,N,N,O,O-TMS
32.6670	34.8505	FERULIC ACID,O,O-TMS
32.7990	34.9844	URIC ACID,N,N,O,O-TMS
32.9920	35.1803	CITRULLINE,O,N,N,N-TMS
33.2270	35.4187	NONADECANOIC ACID ME
33.3370	35.5304	1HO-20:0 TMS
33.7930	35.9931	SORBITOL-6-PHOSPHATE TMS
33.8390	36.0397	FRUCTOSE-6-PPHOSPHATE MEOX1 TMS
34.0000	36.2031	FRUCTOSE-6-PHOSPHATE MEOX2 TMS
34.0560	36.2599	ALLANTOIN,N,N,N-TMS
34.1930	36.3989	GLUCOSE-6-PHOSPHATE MEOX1 TMS
34.2560	36.4629	C19 TMS
34.3770	36.5856	GLUCOSE-6-PHOSPHATE MEOX2 TMS
34.4470	36.6567	CYSTIN TMS
34.6930	36.9063	TRYPTOPHANE,N,N´,O-TMS
34.8760	37.0920	EICOSANOIC ACID ME
34.9220	37.1387	CIS-11-EICOSENOIC ACIDME
34.9840	37.2016	XYLOBIOSE MEOX1 TMS
35.2230	37.4441	XYLOBIOSE MEOX2 TMS
35.2530	37.4745	CIS-11,14-EICOSADIENOIC ACID ME
35.3070	37.5293	CIS-8,11,14-EICOSATRIENOIC ACID ME
35.3960	37.6196	GLUCURONIC ACID-6-PHOSPHATE TMS
35.7670	37.9961	CIS-11,14,17-EICOSATRIENOIC ACID ME
35.7800	38.0093	SINAPIC ACID,O,O-TMS
36.2150	38.4507	TRYPTOPHANE,N,O-TMS
36.4290	38.6678	1HO-22:0 TMS
36.4820	38.7216	HENEICOSANOIC ACID ME
37.1490	39.3984	SUCROSE TMS
37.7170	39.9747	LACTOSE MEOX1 TMS
37.7170	39.9747	LACTOSE MEOX2 TMS
37.9150	40.1757	LACTITOL TMS
37.9870	40.2487	LACTULOSE TMS
38.0060	40.2680	DOCASANOIC ACID ME
38.0710	40.3339	CIS-13-DOCOSENOIC ACID ME
38.2520	40.5176	CELLOBIOSE MEOX1 TMS
38.4010	40.6688	CIS-13,16-DOCOSADIENOIC ACID ME
38.4830	40.7520	MALTOSE MEOX1 TMS
38.6100	40.8809	SACCHAROPINE TMS
38.6550	40.9265	CELLOBIOSE MEOX2 TMS
38.6720	40.9438	TREHALOSE TMS
38.6950	40.9671	LAMINARIBIOSE MEOX1 TMS
38.8950	41.1701	MALTOSE MEOX2 TMS

Observed RT (minutes)	Predicted RT for non-RT-locked data (minutes)	Peak Identity
38.8990	41.1741	TURANOSE MEOX1 TMS
39.0280	41.3050	NIGEROSE MEOX1 TMS
39.0350	41.3121	LAMINARIBIOSE MEOX2 TMS
39.0940	41.3720	MALTITOL TMS
39.3860	41.6683	NIGEROSE MEOX2 TMS
39.4030	41.6855	TURANOSE MEOX2 TMS
39.4940	41.7779	TRICOSANOIC ACID ME
40.2340	42.5287	C23 TMS
40.4790	42.7773	PALATINOSE TMS
40.6890	42.9904	MELIBIOSE MEOX1 TMS
40.8350	43.1386	ISOMALTOSE MEOX1 TMS
40.9260	43.2309	TETRACOSANOIC ACID ME
40.9820	43.2877	MELIBIOSE MEOX2 TMS
41.0180	43.3243	CIS-15-TETRACOSENOIC ACID ME
41.1650	43.4734	ISOMALTOSE MEOX2 TMS
41.9970	44.3177	1HO-26:0 TMS
41.9970	44.3177	1HO-26:0 TMS
42.3040	44.6292	PENTACOSANOIC ACID ME
43.3920	45.7332	SUCROSE-6-PHOSPHATE TMS
43.4310	45.7727	GLUCONIC ACID LACTONE TMS
44.5210	46.8788	1HO-28:0 TMS
45.4510	47.8224	C27 TMS
45.7320	48.1076	1HO-29:0 TMS
46.4410	48.8270	RAFFINOSE TMS
46.8610	49.2532	CHOLESTEROL 1TMS
46.9070	49.2998	1HO-30:0 TMS
47.3750	49.7747	MELEZITOSE TMS
47.6620	50.0659	ALPHA-TOCOPHEROL 1TMS
48.0440	50.4535	1HO-31:0 TMS
48.2670	50.6798	MALTOTRIOSE MEOX1 TMS
48.3420	50.7559	CAMPESTEROL 1TMS
48.5030	50.9193	CELLOTRIOSE MEOX1 TMS
48.6330	51.0512	MALTOTRIOSE MEOX2 TMS
48.7460	51.1659	MALTOTRITOL TMS
48.8630	51.2846	CELLOTRIOSE MEOX2 TMS
49.1540	51.5799	1HO-32:0 TMS
49.3500	51.7787	STIGMASTEROL 1TMS
49.3640	51.7930	BETA-SITOSTEROL 1TMS
49.4750	51.9056	LANOSTEROL 1TMS
50.0630	52.5022	C31 TMS
50.2270	52.6686	1HO-33:0 TMS
51.1380	53.5930	ISOMALTOTRIOSE TMS

10.2.2 ACNFP TMS library

				Match to GMD "Q_MSRI_ID" GC-MS library	
Observed RT (minutes)	Predicted RT for non-RT-locked data (minutes)	Peak Identity	Peak area	Score	Identity of MST match
6.0614	8.2014	Alanine (2TMS)	7.46E+05	94	L-Alanine (2TMS)
7.0463	9.1857	Glycine (2TMS)	1.93E+05	92	Glycine (2TMS)
9.3044	11.4424	Valine (2TMS)	1.59E+07	99	L-Valine (2TMS)
10.3165	12.4539	Cysteine	2.27E+07	33	Indole-3-acetaldehyde {BP} (TMS)
10.9951	13.1320	Leucine (2TMS)	4.37E+06	96	Norleucine (2TMS)
11.8478	13.9842	Glycine (3TMS)	4.05E+07	98	Glycine (3TMS)
12.2585	14.3947	Serine (2TMS)	9.19E+05	95	L-Serine (2TMS)
12.6507	14.7866	Proline (2TMS)	1.51E+05	95	L-Proline (2TMS)
12.9354	15.0711	Threonine (2TMS)	2.41E+06	97	L-Threonine (2TMS)
13.1508	15.2864	Alanine (3TMS)	1.01E+08	98	L-Alanine (3TMS)
13.7530	15.8882	Serine (3TMS)	2.15E+06	99	L-Serine (3TMS)
14.1962	16.3312	Threonine (3TMS)	8.68E+06	97	L-Threonine (3TMS)
14.5209	16.6556	Leucine (3TMS)	3.15E+05	81	L-Leucine (2TMS)
14.9281	17.0626	Maleic acid	2.85E+06	92	Maleic acid (2TMS)
16.2406	18.3743	Erythritol	8.76E+07	96	Erythritol (4TMS)
16.4001	18.5337	Threitol	2.98E+07	98	Erythritol (4TMS)
17.8156	19.9483	Malic acid	1.28E+07	98	Malic acid (3TMS)
17.8666	19.9993	GABA	1.37E+07	97	4-Aminobutyric acid (3TMS)
17.9479	20.0805	Proline 4-Hydroxy	3.45E+05	90	4-Hydroxyproline (3TMS)
18.5887	20.7209	Aspartic acid (3TMS)	5.36E+06	99	L-Aspartic acid (3TMS)
18.7290	20.8611	Asparagine	6.53E+06	94	[L-Asparagine (4TMS)]
19.2333	21.3651	Methionine	5.51E+06	96	L-Methionine (2TMS)
20.2857	22.4169	Tyrosol	9.78E+06	39	4-Hydroxybenzoic acid (2TMS)
20.5711	22.7021	Ribitol	1.73E+07	98	Ribitol (5TMS)
20.7263	22.7343	Arabitol	1.74E+07	96	Xylitol (5TMS)
20.7520	22.7604	Xylitol	1.82E+07	96	Xylitol (5TMS)
21.0649	23.1956	Glutamate	3.63E+07	99	Pyroglutamic acid (2TMS)
21.9230	24.0531	Phenylalanine	7.11E+06	98	L-Phenylalanine (2TMS)
22.4824	24.6122	Proline	8.16E+06	98	[653; L-Proline (2TMS)]
22.9965	25.1260	Asparagine	3.22E+06	46	L-Asparagine (3TMS)
23.1846	25.3140	Cysteine	1.08E+07	81	L-Aspartic acid (3TMS)
24.3133	26.4420	Mannitol	1.84E+07	99	Mannitol (6TMS)
24.5953	26.7238	Fructose Peak A	6.06E+06	98	Fructose methoxyamine (5TMS)
24.6424	26.7709	Mannose	5.68E+06	95	Glucose methoxyamine (5TMS)
24.6520	26.7804	Sorbitol	2.53E+07	99	Mannitol (6TMS)
24.7152	26.8436	Dulcitol	8.62E+07	100	Galactitol (6TMS)
24.8583	26.9270	Galactose A			
24.9280	27.0563	Fructose Peak B	4.35E+06	97	Fructose methoxyamine {BP} (5TMS)
25.1144	27.2426	Glucose Peak A	1.09E+07	94	Glucose methoxyamine (5TMS)
25.1855	27.2590	Galactose B			
25.3603	27.4883	Proline 4-Hydroxy	1.64E+06	69	[646; Proline (2TMS)]
25.3710	27.4990	Glucose Peak B	1.86E+06	97	Galactose methoxyamine {BP} (5TMS)
25.4551	27.5830	Lysine	1.09E+07	90	L-Lysine (4TMS)
25.4716	27.5995	Arginine	7.71E+05	89	L-Arginine (5TMS)
26.2869	28.3766	Allantoin A			
27.5332	29.6412	Allantoin B			
27.8392	29.9657	Tyrosine (3 TMS)	1.95E+07	97	L-Tyrosine (3TMS)
28.9923	31.1180	Hexadecanoic acid	5.78E+05	93	Hexadecanoic acid (1TMS)
29.1305	31.2562	Glutamine (4TMS)	2.05E+05	84	Glutamine (4TMS)
30.3547	32.4796	DOPA	1.85E+06	81	L-Tyrosine (3TMS)
32.4131	34.5367	Octadecanoic acid	1.72E+06	93	Octadecanoic acid (1TMS)
33.2355	35.4274	Mannitol 1-Phosphate			
33.6867	35.8852	Fructose 6-Phosphate A			
33.8529	36.0538	Fructose 6-Phosphate B			
34.0224	36.1450	Nonadecanoic acid	9.48E+05	85	[Nonadecanoic acid (1TMS)]
34.0434	36.2471	Glucose 6-Phosphate A			
34.1834	36.3059	Cystin	3.06E+06	67	S-Methyl-L-cysteine (2TMS)
34.1957	36.4017	Glucose 6-Phosphate B			

				Match to GMD "Q_MSRI_ID" GC-MS library	
Observed RT (minutes)	**Predicted RT for non-RT-locked data (minutes)**	**Peak Identity**	**Peak area**	**Score**	**Identity of MST match**
34.2901	36.4126	Tryptophan	1.45E+06	95	L-Tryptophan (3TMS)
36.9992	39.1200	Sucrose	5.90E+06	96	[N-Acyl-homoserine lactone elicited product]
38.2583	40.3783	Maltose A	1.25E+06	97	[N-Acyl-homoserine lactone elicited product]
38.4630	40.5829	Trehalose	6.48E+06	97	[N-Acyl-homoserine lactone elicited product]
38.6851	40.9571	Maltose B			
40.4751	42.5937	Melibiose	2.15E+06	98	[N-Acyl-homoserine lactone elicited product]
46.0561	48.1712	Raffinose	2.11E+06	95	[N-Acyl-homoserine lactone elicited product]
47.9110	50.3186	Ergosterol			